THE UNIVERSITY OF CALGARY

Bacteriocin Production in *Rhizobium leguminosarum* bv. viciae Strain 306

by

Alexandra Patricia Venter

A THESIS

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ABSTRACT

Rhizobium leguminosarum bv. viciae strain 306 was found to produce three bacteriocins with different activities. A previously uncharacterised bacteriocin with activity against *R. leguminosarum* bv. viciae strain 248 was chosen as the focus of further study. 306 was mutagenised with Tn*5* and two mutants with altered bacteriocin activity against 248 were isolated. The gene regions flanking the mutations were cloned out and the bacteriocin encoding region characterised. 306 was found to produce a novel bacteriocin with sequence homologies to RTX toxins, and to have an ABC transporter system similar to those used by RTX toxins. Production of the bacterocin was not correlated with an improved ability to compete against 248 for nodulation of peas, thus there was no evidence for a role for bacteriocin production by 306 in inter-strain competition under the conditions assayed.

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DEDICATION

For Karim.

Why sometimes I've believed as many as six impossible things before breakfast.

-*The White Queen, in Through the Looking Glass by Lewis Carroll.*

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
Amp	ampicillin
Amp^r	ampicillin resistant
bp	base pairs
Cm	chloramphenicol
Cm^r	chloramphenicol resistant
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'chloro) tricyclo [3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
EPS	exopolysaccharide
$Fix^{+,-}$	fixation plus, minus
fix	nitrogen fixation genes
g	g force
Gm	gentamicin
Gm^r	gentamicin resistant
IS	insertion sequence
Kan	kanamycin
Kan^r	kanamycin resistant
kb	kilobase pairs
kDa	kiloDalton

LIST OF ABBREVIATIONS CONTINUED

Abbreviation	Explanation
L	litre
LPS	lipopolysaccharide
M	molar
min	minute
mL	millilitre
mM	millimolar
µg	microgram
µM	micromolar
nif	nitrogen fixation genes
N	normal
Nm	neomycin
Nm^r	neomycin resistant
nod	nodulation genes
ORF	open reading frame
ori	origin of DNA replication
p	plasmid
$pBSK^+$	pBluescript, ampicillin resistant
$pBC\ SK^+$	pBluescript, chloramphenicol resistant
rpm	rotations per minute
sdH_2O	sterile distilled water
SSC	standard saline citrate

LIST OF ABBREVIATIONS CONTINUED

Abbreviation	Explanation
SDS	sodium dodecylsulfate
SF	supernatant fluid
Sm	streptomycin
Spc	spectinomicin
Spc^r	spectinomicin resistant
Sym	symbiotic genes
Tet	tetracycline
Tet^r	tetracycline resistant
Tn	transposon
vol	volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
°C	degrees Celsius
%	percent

1.0 Introduction

The leading agricultural experts in Ancient Rome were well aware of the beneficial effects of Leguminosae on soil (White 1970). Today this effect is understood to be due to the nitrogen-enriching capacity of these crops: leguminous plants form a symbiotic association with nitrogen-fixing rhizobia, and, in nitrogen poor soils, these root-nodulating bacteria provide an essential source of fixed nitrogen to their host plants (Brewin *et al.* 1992). Much of the world's population, particularly in developing nations, depends on legumes as a source of protein such as that found in beans, lentils, soybeans, cowpeas and chickpeas (Brewin *et al.* 1992). Legume crops can also inexpensively enhance soil fertility without the problems associated with the use of N_2 fertilizers, such as the volatilization of greenhouse gases, the depletion of nonrenewable resources, and the leaching of NO_3^- into the groundwater (Vance 1998). However, indigenous species of rhizobia are often poor at nitrogen fixation, and attempts to inoculate soils with more efficient nitrogen-fixers have failed due to the ability of indigenous rhizobia to out-compete the inoculum strains. Triplett and Sadowsky (1992) have identified this situation as "the *Rhizobium* competition problem."

Various tactics have been proposed to surmount this problem, including the genetic engineering of efficient nitrogen-fixing strains with a competitive advantage, such as the ability to produce antibiotics or bacteriocins (Robleto *et al.* 1998). Alternatively, fields could be co-inoculated with naturally bacteriocin resistant, efficient nitrogen-fixers and bacteriocin producers. Several strains of *Rhizobium leguminosarum* are known to produce such antimicrobial compounds (Hirsch 1979; Lotz and Mayer 1972; Roslycky 1967; Schwinghamer and Belkengren 1968; Schwinghamer 1971; Schwinghamer 1975). The goal of this project was to identify a novel bacteriocin from *R. leguminosarum* bv. viciae 306, clone and characterise the genetic region coding for this bacteriocin, and investigate the role of the bacteriocin in competition between strains.

2.0 Literature Review

2.1 Rhizobia-Legume Symbiosis

The relationship between legumes and rhizobia is characterised by the ability of these Gram-negative bacteria to induce the formation of new plant organs called root nodules, in which the rhizobia fix atmospheric nitrogen (Jordan 1984; Nap and Bisseling 1990). This process involves the activation of both plant and bacterial genes. In the faster growing rhizobia, the bacterial genes for nodulation (*nod*) and nitrogen fixation (*nif* and *fix*) are frequently carried on large symbiotic plasmids called Sym plasmids, although the symbiotic genes can be spread across multiple plasmids and the chromosome within a strain (Finan *et al.* 1986; Hynes *et al.* 1986; Hynes and McGregor 1990; Kamininski *et al.* 1998).

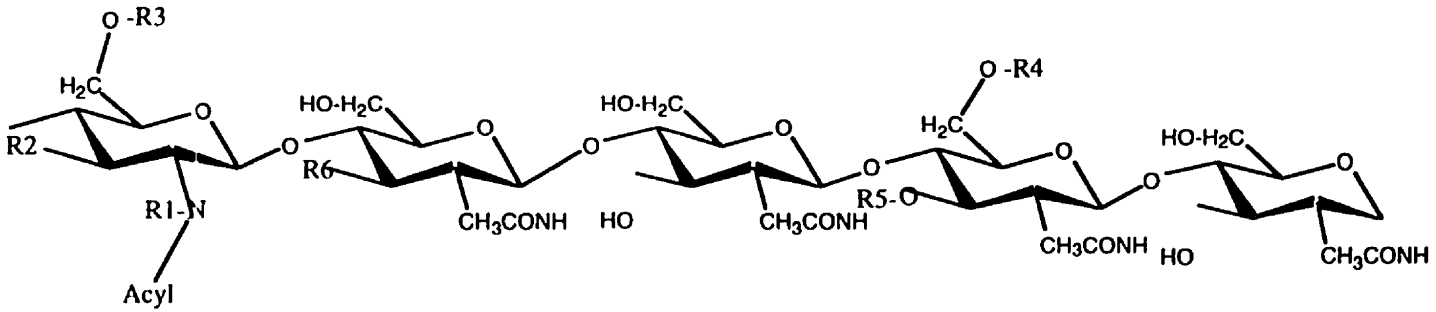
Flavonoids, 2-phenyl-1,4-benzopyrone derivatives, are secreted by plants into the rhizosphere (Perret *et al.* 2000). Flavonoids trigger the induction of bacterial genes required for nodulation, usually in concert with the rhizobial gene product NodD (Schlaman *et al.* 1998). Other inducers of the *nod* genes may include aldonic acids, and trigonelline and stachydrine, nonflavonoids released from seeds (Gagnon and Ibrahim 1998; Phillips *et al.* 1992). NodD proteins are found in the cytoplasmic membrane of *R. leguminosarum* bv. viciae (Schlaman *et al.* 1989). NodD, which is essential for nodulation, acts as a sensor of plant signals and transcriptional activator of the *nod* genes (Nap and Bisseling 1990; Schlaman *et al.* 1992). *nod* boxes are conserved 47-bp motifs located in the promoter region of many nodulation loci. NodD proteins, members of the LysR-like family of transcriptional regulators, bind to the *nod* boxes and, in the presence of flavonoids, induce the nodulation genes (Fisher *et al.* 1988; Rostas *et al.* 1986).

Expression of the nodulation genes results in the production of Nod factors (Perret *et al.* 2000). Nod factors are lipochitooligosaccharides secreted by rhizobia and recognised by the host plant. Nod factor enzymes encoded by *nodABC* are responsible

for the assembly of the Nod factor core (see Figure 1). *nodABC*, which are essential for nodulation, can be found in all strains of *Rhizobium* and are functionally interchangeable between species (Long 1989; Nap and Bisseling 1990; Downie 1998). NodA and NodC are also determinants of host specificity in *Sinorhizobium meliloti* (Kamst *et al.* 1997; Roche *et al.* 1996). NodI and NodJ are involved in the export of Nod factors (Cardenas *et al.* 1996; Spaink *et al.* 1995). Other Nod enzymes facilitate the addition of a variety of substituents onto the Nod factor core (Perret *et al.* 2000). These subtle differences in Nod factor structure determine the host specificity of a given rhizobial biovar. *R. leguminosarum* bv. viciae, for instance, will typically nodulate *Pisum sativum* and *Vicia* spp., but not Phaseoleae or Desmodieae (Table 1)(Perret *et al.* 2000). Nod factors are the so-called "keys" required by rhizobia in order to gain entrance to the legume root (Relic *et al.* 1994).

Nod factors induce the curling of root hairs, unicellular extensions of apical root epidermal cells, as in Figure 2 (Perret *et al.* 2000). Purified Nod factors are capable of inducing root hair curling of specific host plants in the absence of bacteria (Lerouge *et al.* 1990). Rhizobia trapped in a root hair curl then penetrate the root hair at areas of localised plant cell wall hydrolysis and invagination of the plasma membrane (Long 1989). The rhizobia enter the root hair cell in a tubular extension composed of plant cell wall materials, and continue to travel intercellularly up the growing infection thread. NodD1 likely controls genes, in addition to those responsible for Nod factors, which are necessary for the development of the infection thread (Perret *et al.* 2000). Surface polysaccharides including exopolysaccharide (EPS), lipopolysaccharide (LPS), capsular polysaccharides and cyclic ß-glucans are also important in the infection process (Chen *et al.*, 1885; Cheng and Walker,1998; Kannenberg *et al.* 1992; Perret *et al.* 2000). In a recent study using fluorescence microscopy, infection with different EPS mutants resulted in the interruption of root invasion at various stages (Cheng and Walker 1998).

Figure 1. Nod factor. The basic Nod factor core is shown, as well as potential Nod factor extensions (R groups). The subtle differences in Nod factor structure are responsible for host plant specificity of rhizobia. The Nod proteins responsible for creating the various Nod factors are shown. NodX, NodH, and NodZ are involved in the addition of various constituents to fucose (Adapted from Perret *et al.* 2000).



NodS	R1	——	CH_3
NolO	R2	——	H_2NCO
NodL	R3	——	CH_3CO
NodU	R3	——	H_2NCO

NodX	R4	——	CH_3CO
NodH	R4	——	SO_3H
NodZ	R4	——	fucose

NodX	2-O-H_3C
NodH	3-O-O_4S
NodZ	3- or 4-OCH_3C

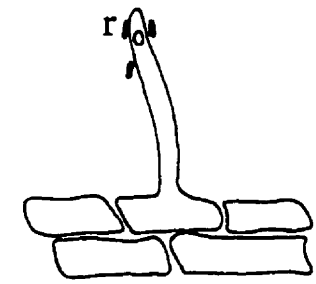
NoeC	R5	——	arabinosyl/ CH_3CO
? NodE NodF NodA	R6	——	CH_3CO
	Acyl	——	various

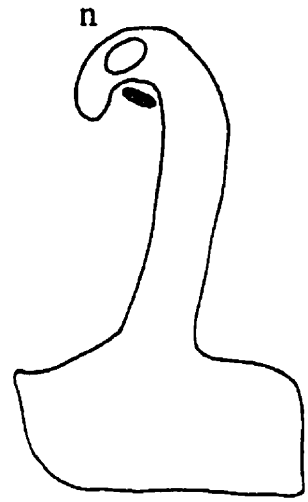
Table 1. Typical Hosts of Selected Rhizobia

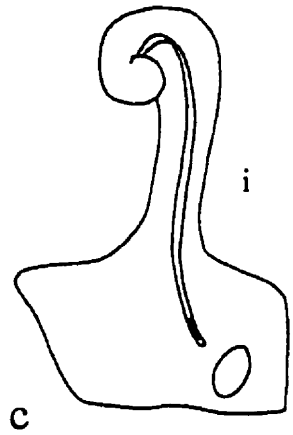
Species	Hosts
Bradyrhizobium japonicum	Phaseoleae, *Glycine* spp.
Mesorhizobium huakuii	*Astragalus sinicus*
Mesorhizobium loti	Loteae, *Lotus* spp., Genisteae, *Lupinus* spp.
Rhizobium etli	Phaseoleae, *Phaseolus* spp.
Rhizobium leguminosarum	
bv. phaseoli	Phaseoleae, *Phaseolus* spp.
bv. trifolii	Trifolieae, *Trifolium* spp.
bv. viciae	Vicieae, *Pisum sativum*, *Vicia* spp.
Sinorhizobium meliloti	Trifolieae, *Medicago* spp., *Melilotus* spp., *Trigonella* spp.
Rhizobium tropici	Phaseoleae, *Phaseolus* spp.

(Adapted from Perret *et al.* 2000)

Figure 2. Infection of Host Plant Leading to Nodule Development. a. Rhizobia (r) are attracted to flavonoids produced by the plant. Here, rhizobia are shown on the root hair surface. b. Nod factors produced by the rhizobia stimulate root hair curling around the bacteria. c. Rhizobia trapped in the root hair curl penetrate the root hair in regions of plant cell wall hydrolysis and invagination of the plasma membrane. The nucleus (n) of the root hair cell precedes the growing infection thread (i), which grows towards the nodule primordia in the root cortex (Adapted from Perret *et al.* 2000).


r
a


n
b


i
c

NodO of *R. leguminosarum* bv. viciae is a 30 kDa secreted protein thought to be involved in infection thread development (Downie *et al.* 2000; Economou *et al.* 1990). NodE is required to produce polyunsaturated acyl groups on Nod factors (Spaink *et al.* 1991). While *nodO* or *nodE* mutants appear to make normal infection threads, double mutants produce thickened or misdirected infection threads (Downie *et al.* 2000). NodO is homologous to haemolysin and other RTX toxins (a family of pore-forming cytolysins), and is capable of forming pores in lipid bilayers (Economou *et al.* 1990; Sutton *et al.* 1994; Welch 1990). Like RTX toxins, NodO can bind Ca^{2+} (Economou *et al.* 1990). NodO may be involved in rhizobia-plant signalling by forming pores in the plant cell plasma membrane (Sutton *et al.* 1994). When cloned into certain rhizobia, *nodO* has also been shown to extend the host range of these strains (Economou *et al.* 1994). NodO is secreted via a Type I secretion complex encoded by *prsDE*, which shows sequence homology to ABC transporters of RTX proteins (Finnie *et al.* 1997). NodO can be secreted by *Escherichia coli* using the *E. coli* HlyBD-tolC secretion system and *Erwinia chrysanthemi* Prt system, and by several rhizobial genera via an uncharacterised secretion mechanism (Scheu *et al.* 1992). While *nodO* is carried on pRL1JI, a symbiosis plasmid, *prsDE* are encoded elsewhere (Scheu *et al.* 1992).

In an effective invasion, the infection thread grows towards the nodule primordia in the root cortex where the rhizobia enter the cytoplasm of the root cortex cells through endocytosis (Nap and Bisseling 1990). Inside the peribacteroid membrane the rhizobial cells differentiate into bacteroids: this unit, composed of bacterial cells and plant derived membrane, is called a symbiosome (Brewin 1998). An indeterminate nodule type, which has a persistent apical meristem, is formed in peas and vetch and may be divided into four zones: the invasion zone; a zone in which plant cells elongate and bacteria multiply; a zone in which the bacteroids fix nitrogen; and, in older nodules, the senescence zone (Nap and Bisseling 1990). Different nodulin genes are expressed by the plant in each zone

(Nap and Bisseling 1990). The nodulin protein leghemoglobin delivers oxygen to the bacteroids and maintains a low concentration of free oxygen, necessary for the function of the nitrogen fixing enzyme, nitrogenase. In addition, the compact arrangement of the nodule parenchyma creates an oxygen diffusion barrier (Nap and Bisseling 1990). The structural genes for nitrogenase are encoded by *nifD*, *nifH* and *nifK* (Kaminski *et al.* 1998). The *nifB*, *nifE* and *nifN* genes are required for the formation of an anaerobic FeMo cofactor of nitrogenase, while NifA activates the transcription of the *nif* genes as well as various other genes in the symbiotic gene region (Nienaber *et al.* 2000; Szeto *et al.* 1984; Zimmerman *et al.* 1983). The *fixNOPQ* operon is thought to code for a respiratory oxidase complex of the bacteroid which is dependent on *fixGHIS* and regulated by *nifA*, *fixK*, *fixT*, and *fixLJ* (David *et al.* 1988; Foussard *et al.* 1997; Preisig *et al.* 1993; Preisig *et al.* 1996; Schlüter *et al.* 1997). Regulation differs somewhat in different rhizobia (Fischer *et al.* 1994). Fix^- strains are unable to fix nitrogen but may be able to form nodules (Nod^+).

2.2 The Competition Problem

Inoculation of soil with highly efficient nitrogen fixing rhizobia is one way of increasing crop yield (Moawad *et al.* 1998). In some cases, inoculation is successful. The inoculation of alfalfa seeds at field sites with recombinant strains of *S. meliloti* increased plant biomass compared to inoculation with the wild type strain (Scupham *et al.* 1996). In addition, the inoculant strains occupied a large proportion of the nodules during the course of the study, about three years. However, nodulation by the inoculant strains was only possible in soil where the indigenous populations were quite low (Scupham *et al.* 1996). As described earlier, the competition problem stems from the fact that indigenous strains often out-compete more efficient nitrogen fixing inoculant strains used in the hopes of improving crop yield (Kucey and Hynes 1989; Hynes and O'Connell 1990;

Moënne-Loccoz *et al.* 1994). For example, in field tests in which the distinctive plasmid profiles of nodulating rhizobia were examined, strains with plasmid profiles matching those of strains used as commercial inoculants over some years were rarely isolated (Hynes and O'Connell 1990). In another test, soil was seeded with an inoculum and the plasmid profiles of nodulating bacteria checked over a four year period (Kucey and Hynes 1989). Again, rhizobia with plasmid profiles matching those of the inoculant strains did not persist in the soil. Some plasmid rearrangements are possible between strains, but extreme changes in plasmid profiles would be unlikely (Moënne-Locoz *et al.* 1994).

2.3 Factors Affecting Competition

Competition refers to the competition between genetically related rhizobia for nodulation of a host plant (Sadowsky and Graham 1998). It can be assumed that mutations affecting most genes required for microbial growth and survival in the soil will also result in a less competitive phenotype, so such genes are not true competition loci (Sadowsky and Graham 1998). As Streeter (1994) points out, studies of the so-called genetic determinants for competition are fraught with limitations, since in the environment, the great genetic diversity of rhizobia are likely able to occupy a variety of niches, and adaptation to survival in some of these niches may not be important for competition for nodulation. Nevertheless, certain factors have been identified which are thought to play more specific roles in competition (Sadowsky and Graham 1998; Triplett and Sadowsky 1992).

2.3.1 Abiotic Factors Affecting Competition

Abiotic factors affecting competition for nodulation include nutrient limitations to plants and bacteria, and soil moisture and organic matter content, type, pH, temperature and texture (Sadowsky and Graham 1998). Variable responses of different isolates of

Rhizobium have been noted to extreme alkaline conditions, temperature increases and increased salt concentrations (Lie 1984; Surange *et al.* 1997). In a Brazilian field study, when acidic soil with low fertility was fertilised with P, K, S, micronutrients and lime, nodulation by an inoculant strain, marked using the *gusA* reporter gene, improved (de Oliveira *et al.* 1998). Strains of clover rhizobia have also shown differences in the ability to resist dessication (Fuhrmann *et al.* 1986). It is postulated that low water activity can alter the types and amounts of root exudates released, as well as plant growth, and thereby indirectly affect the ability of certain strains to compete for nodulation (Sadowsky and Graham 1998). The effect of moisture content and pH will vary depending on the temperature, and extremes in temperature will tend to be detrimental to nodulation (Sadowsky and Graham 1998). Strains with increased tolerance to these various conditions could be expected to compete better than more sensitive strains.

2.3.2 Host Plant Factors

One suggested technique for overcoming the competition problem is the use of specific germplasms of plants which exclude nodulation by undesirable strains of rhizobia (Triplett and Sadowsky 1992). Efficient nitrogen fixers can compete well for nodulation when the host genotype used restricts nodulation by other strains (Cregan and Keyser 1986). In the case of pea cv. Afghanistan, resistance to nodulation by most European strains of *R. leguminosarum* bv. viciae is determined by a recessive gene in the plant (Lie 1984). The *nodX* gene identified in strain TOM allows this strain to overcome the plant's resistance to nodulation (Davies *et al.* 1988). Transfer of *nodX* to normally non-nodulating strains confers the ability to nodulate cv. Afghanistan (Fobert *et al.* 1991).

The type of legume grown can also influence competition by preferential nodulation by certain strains (Hynes and O'Connell 1990). In one study, pea and faba bean plants showed a preference for particular strains of *Rhizobium* (Hynes and

O'Connell 1990). One group of strains, identified by a specific plasmid profile, formed over half of the nodules on pea plants in this field study, while this group was never found in faba bean plants. Conversely, a different group of strains formed most of the nodules on the faba bean plants, but none of the nodules on the pea plants. In a recent Egyptian study, the inoculant strain used was not competitive against the indigenous rhizobia for nodulation of lentils (Moawad *et al.* 1998). However, the inoculant strain was highly competitive against indigenous strains for berseem clover nodulation, and there was a significant improvement in the yield of inoculated clover compared to the uninoculated clover.

2.3.3 Chemotaxis and Motility

Nonmotile mutants of rhizobia not impaired in growth rate or nodulation have been shown to be less competitive for nodulation than their wild type counterparts (Ames and Bergman 1981; Mellor *et al.* 1987). Spontaneous nonmotile mutants which were delayed in nodulation but not initiation of nodulation were less able to adsorb to root hairs than the wild type strain, and were less competitive for nodulation than the wild type strain (Caetano-Anollés *et al.* 1988). Other uncharacterised nonmotile mutants were less competitive than the wild type, while a hypermotile mutant showed enhanced competitiveness (Bauer and Caetano-Anollés 1990; Wei and Bauer 1999). Chemotaxis deficient strains have also been shown to be unsuccessful at competition compared to the wild type strains (Caetano-Anollés *et al.* 1988; Yost *et al.* 1998). Chemotaxis toward specific compounds important in nodulation may be important for a competitive phenotype (Yost *et al.* 1998).

2.3.4 Catabolic Activity

The ability to catabolise certain compounds may give some strains of rhizobia a competitive edge. In a recent study, plasmid encoded genes for the catabolism of rhamnose, sorbitol and adonitol were cloned and mutated (Oresnik *et al.* 1998). Of the three mutants, only the rhamnose mutant was less competitive than the wild type. The genes for rhamnose utilisation are inducible by clover root extracts, which suggests that these genes are important early on in the infection process (Oresnik *et al.* 1998). In another study, the ability to compete well for pea nodulation was correlated with the ability to utilise homoserine, a compound found in abundance in pea root exudate (Hynes and O'Connell 1990). Mimosine, a toxin produced by the tree-legume *Leucaena*, has been found to give mimosine degrading *Rhizobium* a competitive advantage (Soedarjo and Borthakur 1998). Free-living rhizobia able to catabolise rhizopines, which are synthesized in the nodules, are also more competitive than non-rhizopine catabolising strains (O'Connell *et al.* 1996).

Conversely, in a field study in which the gene for inositol use in a *Sinorhizobium meliloti* strain was interrupted by derivatives of the Ω cassette, the mutant strains competed well for nodule occupancy compared to the indigenous population and an unrelated wild type strain (Scupham *et al.* 1996). However, no comparison was made to nodulation by the wild type strain from which the mutants were derived, so it is not possible to determine whether inositol use by the wild type strain is an important characteristic in this setting.

2.3.5 Speed of Nodulation

When roots become infected with rhizobia, an autoregulatory response in the host plant prevents infection by subsequent inoculations (Sargent *et al.* 1987). This response is demonstrated by use of the split-root assay, in which roots of a young plant are

spatially separated. If one section of the roots is inoculated and the other section of the roots inoculated with the same strain of *Rhizobium* more than 24 hours later, nodulation will proceed normally on the first section of roots, but will be retarded on the second section (Sargent *et al.* 1987). Strains which are able to nodulate the host plant quickly could therefore be expected to have a competitive advantage.

A number of studies using genetically unrelated rhizobial strains show that faster nodulation is related to enhanced competitiveness (Malek *et al.* 1998; Stephens and Cooper 1988). A strain of *Rhizobium huakuii* able to nodulate milk vetch faster than *Rhizobium* sp. ACMP18 was more competitive than ACMP18, even when nine times more ACMP18 than *R. huakuii* was used in the inoculum (Malek *et al.* 1998). Conflicting findings show that a strain able to form more early infections than its competitor was ultimately less competitive than the other strain (Zdor and Pueppke 1988). Difficulties in interpreting such studies arise from the fact that "speed of nodulation" is not well defined, and the speed at which initiation of infection can occur may not be related to the speed at which nodules are formed.

2.3.6 Symbiotic Effectiveness

Effective microsymbionts must be able to form nodules on their host and fix nitrogen. A DNA region in *S. meliloti* was identified which was called *nfe* for nodule formation efficiency: mutations in *nfe* delayed nodule formation and reduced competitiveness of the strain (Sanjuan and Olivares 1989). The *nfeC* gene of *B. japonicum* has also been shown to affect competitiveness (Chun and Stacey 1994). A mutation in the *nolJ* gene of *Rhizobium fredii* strain USDA 201 caused a delay in nodulation, a decrease in the efficiency of nitrogen fixation, and a decrease in the competitiveness of the strain (Boundy-Mills *et al.* 1994). Using the split-root assay, Sargent *et al.* (1987) showed that pre-exposure of the roots to a Fix^+ but nodulation

impaired mutant did not prevent later nodulation by the wild type strain. Simultaneous inoculation of one section of the roots with the mutant and the other with the wild type resulted in nodulation blocking of the mutant, and in co-inoculations of a single root assay, the wild type out-competed the mutant for nodulation. However, *R. leguminosarum* bv. viciae TOM is inhibited for nodulating Afghanistan peas by a viciae strain unable to nodulate this plant (Broughton *et al.* 1982). The genes for competitive blocking are located on a Sym plasmid, which confers the competitive blocking phenotype to non-blocking strains (Dowling *et al.* 1987).

Some research indicates that plants select effective nitrogen fixers as symbionts (George and Robert 1991). In contrast, in a study by Hahn and Studer (1986), a Tn*5* induced Fix- mutant of *B. japonicum* competed almost equally well with the isogenic parent strain. Interestingly, more than 60% of the nodules were coinfected. This differs from the normally seen co-infection rate of 2-4 % (Hynes, personal communication). The authors stressed the importance of being able to detect co-nodulation in competition studies (Hahn and Studer 1986).

2.3.7 Bacterial Cell Surface Characteristics

Cell surface characteristics which are host plant determinants or which are essential for nodulation would be expected to be important for competitiveness. The contribution of other cell surface characteristics to a competitive phenotype is not as clear. In the case of some species, EPS is not necessary for effective nodulation. In one study, Tn*5*-induced mutants of *B. japonicum* were obtained which produced EPS with lower molecular weight polysaccharide constituents than EPS from the parent strain (Ozawa *et al.* 1992). The mutants were more competitive than the wild type for the nodulation of soybean (Ozawa *et al.* 1992). However, a different strain of *B. japonicum* was found to be less competitive upon Tn*5*-induced mutation causing EPS-deficiency

(Bhagwat *et al.* 1999). An LPS defective mutant of *S. meliloti* showed altered competitiveness for nodulation of alfalfa in that it was slower to form nodules, produced fewer nodules, and was overall less competitive than the wild type (Lagares *et al.* 1992). This study demonstrates the difficulty in separating the influence of factors necessary for effective nodulation from that of factors solely influencing competition.

2.3.8 Plasmids in *Rhizobium*

Rhizobiaceae typically contain large plasmids. Strains of *R. leguminosarum* have one circular, 3200 kb chromosome and four or more circular plasmids of over 300 kb (Hynes and Finan 1998). While some of these plasmids are required for symbiosis, none is essential for survival and therefore it can be argued that they should not be classified as "mini-chromosomes" (Hynes and Finan 1998). In addition, these genetic elements share traits with smaller plasmids: the rhizobial plasmids can be transferred to other genera and stably replicate, apparently without harming the recipient. It is also possible, although difficult, to cure strains of these large plasmids or delete large portions of them (Hynes and McGregor 1990; Oresnik *et al.* 2000). Functions determined by rhizobial plasmids include symbiotic functions, bacteriocin production (discussed below), catabolic activity and melanin production (Hynes *et al.* 1988; Hynes and Finan 1998). The functions of other plasmids remain unknown (Hynes and Finan 1998). Some plasmids are correlated with a competitive or non-competitive phenotype.

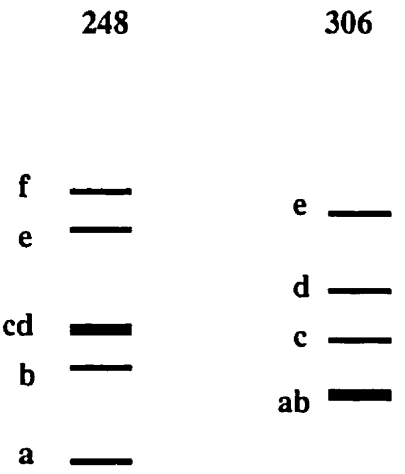
Self-transmissible plasmids were detected in *R. leguminsarum* bv. viciae strains 248, 306 and 309 when these strains were found to transfer bacteriocin production to non-producing strains at frequencies of 10^{-1} to 10^{-2} (Hirsch 1978; Hirsch 1979). The self-transmissible plasmid from 248, pRL1JI, was also found to be a Sym plasmid, and transfer of this plasmid was concomitant with the transfer of the ability to nodulate peas and of other symbiotic functions (Brewin *et al.* 1980; Johnston *et al.* 1978). Transfer of

the plasmids was physically identified on agarose gels by the appearance of extra or larger (rearranged) plasmids in the recipient corresponding to plasmids of the same size in the donors (Hirsch *et al.* 1980). In strain TOM, the transfer of nodulation ability of Afghanistan peas was also found to be encoded on a transmissible plasmid, but this plasmid was not correlated with strain TOM's bacteriocin activity (Brewin *et al.* 1980). The transmissible plasmids from strains 248, 306, 309 and TOM were identified as pRL1JI, pRL3JI (called pRle306b in this study), pRL4JI and pRL5JI respectively (Brewin *et al.* 1980; Hirsch 1979; Johnston *et al.* 1978) (Figure 3). Interestingly, pRL3JI was shown to decrease the symbiotic effectiveness in the host strain (DeJong *et al.* 1981). Other self-transmissible plasmids have no effect on symbiotic effectiveness or competitiveness (Hynes *et al.* 1988).

A number of non-Sym plasmids have been linked to improved competitive behaviour (Brewin *et al.* 1983; Bromfield *et al.* 1985; Martinez-Romero and Rosenblueth 1990). In some cases plasmid curing reduces the ability of a strain to compete with the wild type (Brom *et al.* 1992). On the other hand, plasmid-cured derivatives of *Rhizobium loti* show enhanced competitiveness and nitrogen-fixing efficiency (Pankhurst *et al.* 1986). How competitiveness is influenced by these plasmids warrants further investigation.

In a competitive strain of *Rhizobium etli*, the plasmid encoded *slp* gene is required for a competitive phenotype. Slp, a stomatin-like protein, may be involved in ion transport and play a role in ion exchange and nutrient uptake in the infection thread (You *et al.* 1998).

Figure 3. Schematic Representation of Plasmid Profiles of 248 and 306. Rhizobia have characteristic plasmid profiles as visualised on modified Eckhardt gels (Hynes *et al.* 1985; Hynes *et al.* 1986). In these gels, plasmid DNA migrates in covalently closed circular form. In this representation the smallest plasmids are shown at the bottom of the gel. In lane 248, rhizobial plasmids pRle248a, b (pRL1JI), c, d, e and f are shown. pRle306c and d are shown as a doublet (Hynes, personal communication). pRle248f is not visible in photographs of gels published by Hirsch *et al.* (1980), but can be seen on modified Eckhardt gels (Hynes, personal communication). In lane 306, plasmids pRle306 a and b (pRL3JI) are shown as a doublet, followed by pRle306c, d and e (Adapted from Hirsch *et al.* 1980).


248
306
f
e
cd
b
a
e
d
c
ab

2.3.9 Bacteriocins

Bacteriocins are microbiologically produced proteinaceous compounds which have antibiotic activity only against strains closely related to the producing organism (Barefoot *et al.* 1992). Rhizobiocins refer to bacteriocins produced by *Rhizobium* (Roslycky 1967). The colicins of *E. coli* and other *Enterobacteriaceae* are perhaps the best characterised bacteriocins, but bacteriocin production is known to occur across the spectrum of eubacteria and Archea (Konisky 1982; Sahl and Bierbaum 1998; Jack *et al.* 1995; Cheung *et al.* 1997). The lantibiotics of Gram positive bacteria are of special interest as food preservatives (Jack *et al.* 1995; Sahl and Bierbaum 1998).

It has been said that "substances currently named bacteriocins comprise a rather ill-defined potpourri of proteinaceous molecules . . . " (Jack *et al.* 1995). Indeed, bacteriocins include low molecular weight peptides such as nisin and microcin as well as larger proteins, like the colicins (Jack *et al.* 1995; Konisky 1982; Sahl and Bierbaum 1998). Extracts containing possible bacteriocins can be tested for the presence of phage by diluting preparations of the sample and adding it to lawns of a sensitive indicator strain (Hoover 1992). With increasing dilution, bacteriophage will eventually result in individual plaques, while a bacteriocin will produce shrinking zones of inhibition. In the context of this thesis, bacteriocins do not include the defective phage particles found in some rhizobia (Schwinghamer *et al.* 1973). Defective phage can be detected with electron microscopy. Defective phage can be distinguished from bacteriocins based on the rate of sedimentation of the particles in sucrose gradient centrifugation, and by the UV absorption spectrum of defective phage particles due to the presence of nucleic acids (Schwinghamer *et al.* 1973).

The ability to produce bacteriocins can be a competitive advantage in nature, such as in the ability of *Streptococcus mutans* to colonise the human oral cavity (Hillman *et al.* 1987); the ability of *Leuconostoc plantarum* to compete in green olive fermentation vats

(Riley 1998); and the ability of haemocin producing strains of *Haemophilus influenzae* to kill most other non-type b *H. influenzae* strains (Lipuma *et al.* 1990). Bacteriocin production is also thought to play a role in colonisation of the rumen by microorganisms such as *Butyrivibrio* (Kalmokoff and Teather 1997). Bacteriocins may be a key factor in the mediation of population dynamics (Riley 1998).

In comparison to the colicins, microcins and lantibiotics, little is known about the structure and function of rhizobiocins.

2.3.9.1 Trifolitoxin

Work by the Triplett research group has focussed on the practical application of a rhizobiocin to the *Rhizobium* competition problem (Triplett and Barta 1987; Triplett 1988; Triplett 1990; Triplett and Sadowsky 1992; Scupham *et al.* 1996; Robleto *et al.* 1997; Robleto *et al.* 1998). *R. leguminosarum* bv. trifolii strain T24 is known to induce ineffective nodules on clover roots and prevent nodulation by other rhizobia (Schwinghamer and Belkengren 1968). T24 is able to out-compete other *Rhizobium* strains due to its production of trifolitoxin (TFX), an inhibitory anti-rhizobial antibiotic (Triplett and Barta 1987). TFX, which is produced constitutively, has a relatively wide range of activity (Robleto *et al.* 1998; Triplett and Barta 1987).

Triplett and Barta (1987) have provided evidence that TFX increases the competitiveness for nodulation by T24. In their study, a Tn*5* mutant of T24 (Tn*5*-1) was created that was resistant to, but no longer produced TFX. Another Tn*5* mutant (Tn*5*-4) was deficient in nodulation. It was shown that T24 or Tn*5*-4 could prevent the nodulation of alfalfa by a TFX-sensitive strain of *S. meliloti*, if another, effectively-nodulating TFX-resistant strain of *S. meliloti* were present. T24, Tn*5*-1 and Tn*5*-4 in combination, could also decrease the nodulation of clover by an effective TFX-sensitive strain of *R. leguminosarum* bv. trifolii.

The genes for trifolitoxin production and resistance have been isolated and sequenced (Triplett 1988; Triplett *et al.* 1989; Breil *et al.* 1993). A 7.1 kb fragment of T24 DNA which confers trifolitoxin production and resistance to nonproducing strains has seven complete open reading frames, *tfxABCDEFG* (Triplettt *et al.* 1989; Breil *et al.* 1993). Deletions in *tfxA* and part of *tfxB* result in a loss of the competitive phenotype (Robleto *et al.* 1997). The predicted structure of TfxA, the structural backbone, indicates that it may encode a leader sequence commonly found in ribosomally synthesized prepeptides (Breil *et al.* 1993; Triplett 1999). *tfxG* and possibly *tfxE* are required for resistance to TFX, and by using Tn*3*GUS mutations to interrupt the *tfx* genes, it has also been shown that TfxA, D and F and possibly B and C are required for trifolitoxin production (Breil *et al.* 1993). TfxD may be involved in export, and TfxA, B, and F are required for post-translational modifications of the peptide (Triplett 1999). The *tfuA* gene is also important for TFX production in T24, but this gene may be a housekeeping gene found in all related strains (Breil *et al.* 1996). The predicted amino acid sequence of TfxB shares 27.6% identity with the McbC protein required for the production of microcin B17 (Breil *et al.* 1993). Like the microcins, TFX is a low molecular weight peptide (11 amino acids), which sets it apart from the medium sized rhizobiocins (Hirsch 1979). Unlike the medium rhizobiocins, TFX appears to be chromosomally encoded (Triplett 1988).

Two important aspects of the creation of recombinant bacteriocin producing strains include the effect of transferred bacteriocin genes on the recipient, and their ability to persist in this strain. First, the TFX genes must increase the ability of the recipient to compete for nodulation in the soil. Furthermore, the introduction of bacteriocinogenic genes into an effective-nodulating strain must not reduce nitrogen fixation efficiency if the new strains are to be used in agriculture. pTFX1, a genetically engineered plasmid carrying the TFX gene region, restores nodulation competitiveness to a TFX-minus T24

mutant, and confers TFX production to other, nonproducing strains of *Rhizobium* and *Agrobacterium* (Triplett 1988). Active TFX can be partially purified from T24 and other strains carrying pTFX1 using reverse-phase and anion exchange chromatography (Triplett 1988). The *tfxABCDEFG* gene cassette increases the ability of *Rhizobium etli* CE3 to compete for rhizosphere colonisation and root nodulation in *Phaseolus vulgarus* in sterile and non-sterile soil (Robleto *et al.* 1997). These genes also increase the competitiveness of CE3 for root nodulation in the field, without adversely affecting bean yield (Robleto *et al.* 1998). In addition, the TFX gene region does not affect nitrogenase activity in *R. leguminosarum* bv. trifolii TA1 (Triplett 1990). Unfortunately, *Bradyrhizobium japonicum*, an important soybean symbiont in North America, is not sensitive to TFX, so TFX production cannot be used to enhance the competitiveness of this species compared to indigenous *B. japonicum* strains (Triplett 1999; Triplett and Barta 1987).

In order for bacteriocin producing strains to be of practical use it is also important that plasmids carrying the bacteriocin genes are not lost from recombinant strains in the field, where the application of antibiotics in order to maintain selection pressure is not feasible (Triplett 1990). Stable transfer of the TFX genes to an effective *Rhizobium* strain has been achieved by marker exchange with the recipient chromosome (Triplett 1990). The use of a recombinant plasmid carrying *tfxABCDEFG* and having a plasmid partitioning locus from a broad-host range plasmid also maintains these genes in the recipient (Robleto *et al.* 1997).

While TFX production improves the competitiveness of *Rhizobium*, there are a number of potential drawbacks associated with the production of TFX in the field. Of primary concern is the negative effect of TFX on the biodiversity of the rhizosphere (Robleto *et al.* 1998). Robleto *et al.* (1998) have investigated the effect of TFX on the range of bacterial species present in the rhizosphere by amplifying 16SrRNA-23SrRNA

intergenic regions from DNA isolated from an environmental sample. TFX production in the rhizosphere results in a drastic reduction in the species of α-*Proteobacteria* normally present in the rhizosphere. The profile for the total microorganism population was not substantially altered, however, indicating that TFX has little effect on most other microorganisms (Robleto *et al.* 1998). Nevertheless, such an extreme effect on biodiversity is undesirable, and since TFX has such a wide range of activity, it could become very difficult to eliminate TFX producers from the environment if need be. The introduction of other useful strains to the field could become impossible. In addition, the use of TFX in the field may result in the development of TFX resistance in undesirable species and strains. It therefore seems prudent to investigate the use of a variety of bacteriocins in the field.

Other rhizobiocins, such as the medium bacteriocins produced by *R. leguminsarum* bv. viciae strains 248, 306 and 309, and bv. trifolii strain 162Y10, are only effective against closely related strains and would therefore not be expected to greatly affect the biodiversity of the rhizosphere. However, the techniques used to isolate the *tfx* genes and their application in competition studies are relevant to the study of the medium bacteriocins.

2.3.9.2 Small and Medium Bacteriocins

Hirsch (1979) delineated two classes of bacteriocins produced by *R. leguminosarum*: small bacteriocins were heat labile, diffused through cellophane, and produced wide zones of inhibition (10 to 25 mm) on indicator plates. Ten of the small bacteriocin producers were tested for cross-resistance, and each was found to be resistant to the small bacteriocins produced by the other strains. All of the 83 small bacteriocin producing isolates were also found to be resistant to small bacteriocins produced by 2 other strains. Small bacteriocins are chloroform soluble and easily purified from cell free

supernatants of producing strains (Hirsch 1979; van Brussel *et al.* 1985). The second class of rhizobiocins identified by Hirsch (1979), medium bacteriocins, were heat labile, did not diffuse through cellophane, and produced small zones of inhibition (2 to 10 mm) on indicator plates. Chloroform treated cell-free supernatants from medium bacteriocin producers were found to retain bacteriocin activity even after several weeks at 4°C (Hirsch 1979). Of the medium producers tested, all isolates were resistant to their own bacteriocins, but there was little cross resistance among them. *R. leguminosarum* bv. viciae 336, a small bacteriocin producer, was found to be sensitive to all or most medium producers tested, and was therefore chosen as an indicator strain for medium bacteriocin production.

R. leguminosarum bv. viciae strains 248, 306, and 309, were found to transfer medium bacteriocin production to a non-producing strain via conjugative plasmids, pRL1JI (a Sym plasmid), pRL3JI and pRL4JI respectively (Hirsch 1979). The ability to produce small bacteriocins was lost from small producers following the introduction of these medium bacteriocinogenic plasmids (Hirsch 1979). When pJB5JI, a derivative of pRL1JI carrying Tn*5* in the bacteriocin genes, was introduced into a small producer, small bacteriocin production was again lost from the recipient strain (Beringer *et al.* 1978; Brewin *et al.* 1980; Johnston *et al.* 1978). Wijffelman *et al.* (1983) found that several strains that did not produce small bacteriocins carried the genes for small production, but that this function was repressed. The genes for the repression of small bacteriocin production were located to self-transmissible plasmids, including pRL1JI, not found in small bacteriocin producers. Small bacteriocin resistant mutants with mutations in pRL1JI were also Tra-, indicating that the *tra* genes and genes for repression of small bacteriocin production (*rps*) were spatially very close (Priem, personal communication to Wijjfelman *et al.* 1983).

Small bacteriocins have now been identified as autoinducers of the *N*-acylated

homoserine lactone-type (AHL) (Gray *et al.* 1996; Schripsema *et al.* 1996). Small bacteriocins are therefore structurally related to the quorum sensing co-transcription factors produced by bacteria such as *Pseudomonas aeruginosa*, *Vibrio fischerii* and *Agrobacterium tumefaciens* (Schripsema *et al.* 1996). It has been determined that the small sensitivity region codes for two tandem LuxR-like regulators, BisR and TriR (Downie, personal communication). When transferred to strains cured of pRL1JI, *bisR* and *triR* repress the production of small bacteriocin and confer sensitivity to smalls (Downie, personal communication). These genes are thought to act by repressing the locus for small bacteriocin production (Downie, personal communication).

The 248 bacteriocin encoding region on pRL1JI has been cloned and sequenced (Oresnik *et al.* 1999). Bacteriocin activity is conferred by a 3 kb region, which contains part of the bacteriocin open reading frame. Tn*5* inserts in the 3' half of the 2.8 kb open reading frame do not cause a loss of activity (Oresnik *et al.* 1999). The bacteriocin encoding region shows substantial homology to RTX toxins, as described in the following section (Oresnik *et al.* 1999). Sequence analysis and SDS polyacrylamide gel electrophoresis (PAGE) data suggest that the protein is 102 kDa, and its predicted amino acid sequence reveals a number of hydrophilic domains (Oresnik *et al.* 1999). A cryptic 19-25 amino acid motif is periodically repeated six times every 127 to 132 amino acids within the open reading frame (Oresnik *et al.* 1999). The same motif was found in a bacteriocin encoding region of *R. leguminosarum* bv. trifolii 162Y10 (Twelker *et al.*, unpublished). No specific transporter for the bacteriocin from 248 has been identified (Twelker *et al.* 1999).

The gene region for a novel bacteriocin produced by *R. leguminosarum* bv. trifolii 162Y10 has also been identified and isolated in the Hynes laboratory (Twelker *et al.* 1999). The entire region is over 18 kb long (Twelker and Hynes, unpublished; Accession numberAF141932). Like the 248 bacteriocin gene region, the bacteriocin encoding region

from 162Y10 shows homology to RTX toxins and, in addition, to ABC transporters, although the two bacteriocin encoding regions have little overall homology to one another (Twelker *et al.* 1999). The size of the bacteriocin from 162Y10 is estimated to be 438 kDa (Twelker and Hynes, unpublished). The predicted amino acid sequence suggests a protein of more than 4000 amino acids (Twelker and Hynes, unpublished).

Medium bacteriocin producing strains 306 and 309 have the same range of bacteriocin activity and have very similar plasmid profiles on an Eckhardt gel (Hirsch *et al.* 1980; Hynes, personal communication). These strains also appear to produce a bacteriocin which is very similar to that produced by 248 (Hirsch 1979; Oresnik *et al.* 1999). A Southern blot of genomic DNA from *R. leguminosarum* strains 248, GF160, 306, 162Y10, W14-2, and 309 was probed with a 5.2 kb DNA fragment from the bacteriocin encoding region of 248. The DNA from 248 hybridised only with DNA isolated from itself, and strains 306 and 309 (Oresnik *et al.* 1999). The size of the three hybridising bands were the same, regardless of the restriction enzyme used to digest the genomic DNA. The bacteriocin probe also hybridised to pRL1JI, pRL3JI (pRle306b) and pRL4JI (pRle309b) on a blot of an Eckhardt gel of 248, 306 and 309 DNA (Oresnik, personal communication).

Strain 248 is inhibited by 306 and 309, while 306 and 309 are resistant to 248 (Table 2) (Hirsch 1979). This evidence suggests that 306 and 309 produce another medium bacteriocin in addition to that which is also produced by 248. Furthermore, 336/pJB5JI gives partial resistance against 248, but none against 306 and 309 (Table 3) (Hirsch 1979). Also, the insertion of pJB5JI into 248, 306 and 309 in order to force the loss of incompatible bacteriocinogenic plasmids results in total loss of bacteriocin production from 248, but not from 306 or 309 (Table 3). In further tests, Hirsch (1979) found that a former non-producing strain (1062) carrying bacteriocinogenic plasmids

Table 2. Cross Resistance of Bacteriocin Producing *Rhizobium leguminosarum* Strains

	Indicator Strain		
Test Strain	306	309	248
306	-	-	+
309	-	-	+
248	-	-	-

- no inhibition; + inhibition (Hirsch 1979)

Table 3. Medium Bacteriocin Production and Resistance in Strains Carrying Bacteriocinogenic Plasmids[a]

	Indicator Strain	
Producing Strain	336	336pJB5JI
1062 (control)	-	-
248	+	+/-
306	+	+
309	+	+
1062pRL1JI	+	-
1062pRL3JI	+	-
1062pRL4JI	+	-
1062pJB5JI	-	X
248pJB5JI	-	X
306pJB5JI	+	X
309pJB5JI	+	X

[a] pRl1JI, pRl3JI and pRL4JI are bacteriocinogenic plasmids from *Rhizobium leguminosarum* bv. viciae strains 248, 306 and 309 respectively. pJB5JI carries *Tn*5 in the bacteriocin region on pRl1JI (Johnston *et al.* 1978).

- no inhibition; +/- partial inhibition; + clear zone of inhibition; X not tested (Hirsch 1979)

pRL1JI, pRL3JI, or pRL4JI inhibited 336 but not 336/pJB5JI. These results indicate that the additional bacteriocin produced by 306 and 309 is not carried on pRL3JI or pRL4JI (Hirsch 1979). This bacteriocin encoding region has since been located to pRle306c, but has not been further characterised (Twelker and Hynes, unpublished).

2.3.9.3 RTX Toxins and Homologies to Rhizobiocins

RTX toxins are calcium-dependent, pore-forming cytotoxins produced by various Gram negative pathogens (Welch 1991). These toxins are secreted via ABC transporters (Welch 1991). Well characterised RTX toxins include the haemolysin of *E. coli*, leukotoxin of *Pasteurella haemolytica*, adenylate cyclase of *Bordetella pertussis*, and RTX toxins of *Actinobacillus pleuropneumoniae* (Welch 1991). The common structural motif for which RTX toxins are named is a "repeat in toxin," which is a tandem array of nine amino acids in each toxin protein (L-X-G-G-X-G-(N/D)-D-X) (Welch 1991). The gene organisation for the production of an *E. coli* haemolysin is depicted in Figure 4 (Hess *et al.* 1986). HlyA, the structural component of the RTX toxin, requires activation by HlyC, and is secreted by the action of HlyB, HlyD and the outer membrane factor TolC (Figure 5) (Fath and Kolter 1993; Felmlee *et al.* 1985).

HlyB and HlyD are part of an ABC transport system. ABC transporters are ancient transporters found in eubacteria, eukaryotes and Archaea (Saurin *et al.* 1999). These transporters are characterised by the presence of an ATP binding cassette which is thought to couple ATP, a primary energy source, with translocation (Koronakis *et al.* 1988). Proteins secreted via ABC transport systems do not have cleavable N-terminal signal peptides; instead, the translocation complex recognises an export signal in the C-terminus which is not cleaved (Duong *et al.* 1996). HlyB, the ABC protein in the haemolysin secretion complex, is thought to span the inner membrane. The accessory protein HlyD most likely spans the periplasm, anchored in the inner membrane via its

Figure 4. Generalised Genetic Organisation of RTX Toxins. The key features of typical RTX toxin operons are shown, including the approximate location of the calcium binding repeats found in the structural genes for RTX toxins, and the ABC in the transporter. Based on the haemolysin model, the first gene in the operon is *hlyC*, which codes for the activator of the RTX toxin encoded by *hlyA*. The toxin transporters are coded for by *hlyB* and *hlyD*. The *tolC* gene product, which lies distal to the operon, is also necessary for secretion of HlyA in *E.coli* (Adapted from Hess *et al.* 1986 and Welch 1995).

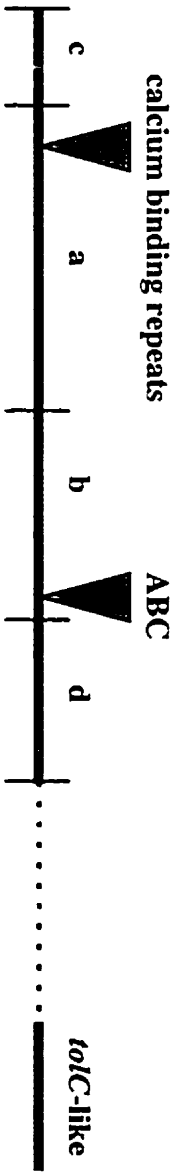
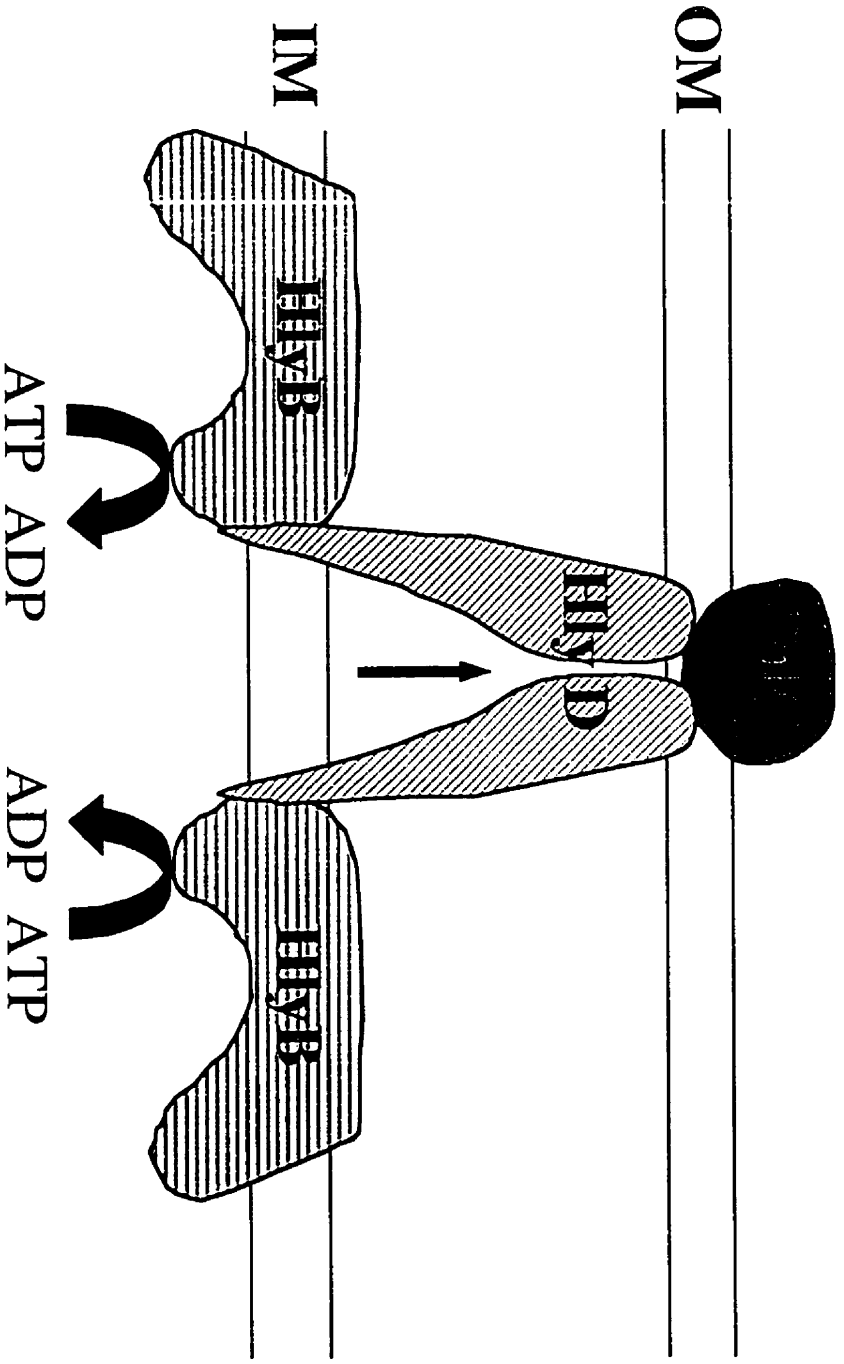

calcium binding repeats
c
a
b
d
ABC
tolC-like

Figure 5. Model for the Secretion of HlyA. Active HlyA is secreted via the action of HlyB, HlyD and TolC. HlyB is the ABC transporter, and HlyD, which spans the periplasm, is an accessory protein which aids in secretion. TolC is an outer membrane factor which is also necessary for the secretion of HlyA in *E.coli*. The inner membrane (IM) and outer membrane (OM) are shown (Adapted Fath and Kolter 1993).


OM
IM
EttyB
EttyB
ATP ADP
ADP ATP

single transmembrane domain (Fath and Kolter 1993). TolC, an accessory outer membrane factor, is also required for HlyA secretion (Felmlee *et al.* 1985).

The bacteriocin encoding region from 248 shows substantial sequence similarities to RTX toxins (Oresnik *et al.* 1999). In bacteriocin tests, the 248 protein has also shown calcium dependence (Oresnik *et al.* 1999). Sequence analysis of the bacteriocin gene region from 162Y10 reveals homologies to the calcium binding region of RTX toxins (Welch 1991; Twelker *et al.* 1999). In addition, the bacteriocin region contains two open reading frames, *rspD* and *rspE*, which encode proteins with substantial homologies to ABC transporters of RTX toxins, and even stronger homologies to PrsD and PrsE, the proteins involved in secretion of NodO in *R. leguminsarum* bv. trifolii (Finnie *et al.* 1997; Håvarstein *et al.* 1995; Twelker *et al.* 1999). Tn*5*B20 inserts in either gene result in a loss of bacteriocin activity and the absence of the > 120 kDa band otherwise seen in SDS PAGE gels (Twelker *et al.* 1999). It therefore seems that the 162Y10 bacteriocinogenic region may contain genes for an ATP-binding export protein and a toxin related to the RTX family (Twelker *et al.* 1999). The similarities between the rhizobiocins and the RTX toxins may indicate that the rhizobiocins have cytolytic activity, although given the vast differences between prokaryotic and eukaryotic cell structure it is not clear how this effect would be achieved. It is not known whether the medium bacteriocins have pore forming activity against plant cells, where membrane disruption of the root cells could be an advantage to rhizobia in the establishment of an infection thread.

2.3 Objectives and Hypothesis

R. leguminosarum bv. viciae 306 is a medium bacteriocin producer of more than one bacteriocin (Hirsch 1979). We hypothesise that bacteriocin production by 306 affords this strain a competitive advantage over closely related bacteriocin sensitive strains for the nodulation of host plants. We predict that the bacteriocin activity of 306

is in part due to a previously uncharacterised bacteriocin which may have structural similarities to bacteriocins produced by *R. leguminosarum* bv. viciae strain 248 and bv. *trifolii* strain 162Y10. The objectives of this project were to clone and characterise the genetic region coding for a novel bacteriocin from 306, and to examine the role of this bacteriocin in competition between strains.

3.0 Materials and Methods

3.1 Growth and Storage of Bacterial Cultures

Antibiotics were added to broth and solid media as required for the maintenance of plasmids, or maintenance of or selection for antibiotic resistance in certain strains. For concentrations of antibiotics used see Appendix A.

Escherichia coli strains were typically grown overnight in LB or TY broth media at 30°C on a roller. Plated cultures were grown on LB plates at 37 °C overnight. Recipes for media and solutions are given in Appendix A.

Agrobacterium tumefaciens and *Sinorhizobium meliloti* strains were typically grown overnight in LB or TY broth media at 30 °C on a roller. Plated cultures were grown on LB or TY plates at 30 °C for two or more days.

Rhizobium strains were grown for 1 to 3 days in TY broth at 30 °C on a roller. Plated cultures were grown on TY or VMM plates at 30 °C for two or more days. For long term storage, cultures were grown to log phase and frozen at -80 °C in 17-25 % glycerol in TY or LB broth.

All strains and plasmids used are listed in Tables 4 and 5.

3.2 Tests for Bacteriocin Activity

3.2.1 Stab Method

This method was adapted from that described by Oresnik *et al.* (1999). Indicator strains were grown up for 12 to 36 hours (to an approximate concentration of 10^8 cells/mL). These strains were then diluted to about 1/25-1/500 in TY broth or sterile water. 1 mL of each diluted culture was used with approximately 20 mL of molten soft TY to make each indicator plate. For large scale screening for Tn*5* bacteriocin$^-$ mutants, indicator strains were added directly to the medium and the plates were then poured.

Test strains were taken from fresh plates and stabbed into the solidified indicator

Table 4. Bacterial Strains

Strain	Relevant Characteristics	Reference/Source
Agrobacterium spp		
ChAg4	wt	Sawada and Ieki 1992
Kag3	wt	Sawada and Ieki 1992
A. tumefaciens		
UBAPF2	plasmid free strain	Hynes *et al.* 1985
Escherichia coli		
DH5α	plasmid free strain, F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44* λ^- *thi*-1 *gyrA96 relA1*	GIBCO-BRL
J53	carries RP4-4, F^- λ *pro met*	Jobanputra and Datta 1974
MM294a/pRK602	*pro82 thiI hsdR17 supE44* with suicide vector pRK602	Finan *et al.* 1986
MT616	MT607/pRK600, *recA56 pro82 thiI hsdR17 supE44*	Finan *et al.* 1986
S17-1 /pSUP102Gm::Tn*5*B20	carries RP4-2Tc::Mu Km::Tn7 in chromosome, *rec*$^-$ *hsdR pro mod*$^+$ *res*$^-$ with pSUP102Gm::Tn*5*B20	Simon *et al.* 1989

Table 4. Bacterial Strains Continued

Strain	Relevant Characteristics	Reference/Source
Rhizobium etli		
Brazil 5	wt	Hynes lab collection
CFN42	wt	Rosenblueth *et al.* 1998
F8	wt	Rosenblueth *et al.* 1998
Viking 1	wt	Hynes lab collection
1-3	wt	Hynes lab collection
Rhizobium leguminosarum		
bv. phaseoli, 4292	Rf^r	Lamb *et al.* 1982
bv. phaseoli, 8401/pJB5JI	8401 with Tn5 insertion in bacteriocin gene region on pRl1JI	Downie collection
bv. trifolii, W14-2	Sm^r	Baldani *et al.* 1992
bv. trifolii, 162Y10	wt	Moënne-Loccoz *et al.* 1994
bv. viciae, B157	Rf^r	Brewin 1982
bv. viciae, GF160	Sm^r	Finan *et al.* 1981
bv. viciae, VF39SM	Sm^r wt	Priefer 1989
bv. viciae, 248	wt	Hirsch 1979
248/pJB5JI	bacteriocin$^-$ 248 strain with Tn*5* insertion in pRL1JI	Beringer *et al.* 1978
248/pRP4-4	wt 248 with helper plasmid RP4-4	This study

Table 4. Bacterial Strains Continued

Strain	Relevant Characteristics	Reference/Source
Rhizobium leguminosarum		
bv. viciae, 336	wt	Hirsch 1979
bv. viciae, 3841	Sm^r derivative of 300	Poole *et al.* 1994
bv. viciae, 309	wt, Fix^-	Hirsch 1979
309SM	Sm^r 309 isolate, Fix^-	Twelker, unpublished
309DM	wt 309, Fix^+	Bristol collection
bv. viciae, 306	wt, Fix^-	Hirsch 1979
306SM	Sm^r 306 isolate, Fix^-	Twelker, unpublished
306DM	wt 306, Fix^+	Bristol collection
M1	bacteriocin$^-$ 306SM Tn*5* mutant	This study
M2	bacteriocin weak 306SM Tn*5* mutant	This study
Ω1, Ω2, Ω4, Ω8	306SM bacteriocin$^-$ mutants with Ωspc cassette insertions	This study
Ω12	306DM bacteriocin$^-$ mutant with Ωspc cassette insertion	This study

Table 4. Bacterial Strains Continued

Strain	Relevant Characteristics	Reference/Source
306SM Tn-*mob-sac* mutants[a]		
pRle306a::Tn-*mob-sac*55	Tn-*mob-sac* insertion in pRle306a	Twelker, unpublished
pRle306b::Tn-*mob-sac*12	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306b::Tn-*mob-sac*30	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306b::Tn-*mob-sac*32	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306b::Tn-*mob-sac*38	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306b::Tn-*mob-sac*59	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306b::Tn-*mob-sac*73	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306b::Tn-*mob-sac*94	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306b::Tn-*mob-sac*95	Tn-*mob-sac* insertion in pRle306b	Twelker, unpublished
pRle306c::Tn-*mob-sac*35	Tn-*mob-sac* insertion in pRle306c	Twelker, unpublished
pRle306c::Tn-*mob-sac*63	Tn-*mob-sac* insertion in pRle306c	Twelker, unpublished
pRle306c::Tn-*mob-sac*69	Tn-*mob-sac* insertion in pRle306c	Twelker, unpublished
pRle306d::Tn-*mob-sac*57	Tn-*mob-sac* insertion in pRle306d	Twelker, unpublished
pRle306d::Tn-*mob-sac*61	Tn-*mob-sac* insertion in pRle306d	Twelker, unpublished
pRle306e::Tn-*mob-sac*90	Tn-*mob-sac* insertion in pRle306e	Twelker, unpublished

[a]306SM Tn-*mob-sac* mutants created by Twelker (unpublished) were further characterised in this study. The plasmid in which the transposon was inserted in each mutant was reconfirmed (mutants pRle306c::Tn-*mob-sac*63, pRle306c::Tn-*mob-sac*69, pRle306d::Tn-*mob-sac*57, pRle306d::Tn-*mob-sac*61, pRle306e::Tn-*mob-sac*90) or determined (remaining mutants listed) in this study.

Table 4. Bacterial Strains Continued

Strain	Relevant Characteristics	Reference/Source
Rhizobium tropici		
CFN299	wt	Rosenblueth *et al.* 1998
CIAT899	wt	Graham *et al.* 1982
Sinorhizobium meliloti		
AK631	wt	Hynes lab collection
cc2013	wt	Hynes lab collection
ML1	wt	Charles lab collection
ML2	wt	Charles lab collection
ML3	wt	Charles lab collection
ML4	wt	Charles lab collection
ML5	wt	Charles lab collection
RCR2012	wt	Hynes lab collection
Rm1021	SU47 *str*-21	Meade *et al.* 1982
102F34	wt	Hynes lab collection
102FS1	wt	Hynes lab collection
104A14	wt	Hynes lab collection
Br816	wt	Hynes lab collection

Table 5. List of Plasmids

Plasmid	Relevant Features	Reference/Source
Rhizobial plasmids		
pRle306a	306 plasmid	Hirsch *et al.* 1980
pRle306b	bacteriocin producing 306 plasmid	Hirsch *et al.* 1980
pRle306c	bacteriocin producing 306 plasmid	This study
pRle306d	306 plasmid	Hirsch *et al.* 1980
pRle306e	306 plasmid	Hirsch *et al.* 1980
pJB5JI	pRL1JI from 248 with Tn*5* insertion	Beringer *et al.* 1978
Cloning vectors and helper plasmids		
pBC SK$^+$	cloning vector, Cm^r, MCS in *lacZ* flanked by T3 and T7	Stratagene Cloning Systems 1995
pBSK$^+$	cloning vector, Amp^r, MCS in *lacZ* flanked by T3 and T7	Stratagene Cloning Systems 1995
pHP45Ωspc	carries Ωspc cassette	Prentki and Krisch 1984
pJQ200SK$^+$	gene replacement vector, Gm^r, MCS in *lacZ*, and *sac*B	Quandt and Hynes 1993
pRK600	helper plasmid	Finan *et al.* 1986
pRK602	suicide vector	Finan *et al.* 1986
pRK7813	cosmid vector, Tet^r, cloning site in *lacZ*, capable of replication in *Rhizobium*	Jones and Gutterson 1987

Table 5. List of Plasmids Continued

Plasmid	Relevant Features	Reference/Source
pUC18	cloning vector, Amp^r	Norrander *et al.* 1983
pUC1918	pUC based cloning vector, Amp^r, MCS in *lacZ*	Schweizer 1993
RP4-4	helper plasmid	Hedges and Jacob 1974
Constructs		
pAVM1	*Eco*RI fragment with Tn*5* insert from M1, in $pBSK^+$	This study
pAVJQM1	*Xba*I fragment from pAV1819M1, in $pJQ200SK^+$	This study
pAVRKM1	*Eco*RI fragment with Tn*5* insert from M1, in pRK7813	This study
pAV1819M1	*Eco*RI fragment with Tn*5* insert from M1, in pUC1819	This study
pAVM1BamKm	*Bam*HI subclone of pAVM1, in $pBC\ SK^+$	This study
pAVM1BamSm	*Bam*HI subclone of pAVM1, in $pBSK^+$	This study
pAVC3	*Cla*I subclone of pAVM1BamSm, in $pBSK^+$	This study

Table 5. List of Plasmids Continued

Plasmid	Relevant Features	Reference/Source
pAVS2	*Sal*I subclone of pAVM1BamSm, in pBSK$^+$	This study
pAVS4	*Sal*I subclone of pAVM1BamSm, in pBC SK$^+$	This study
pAVS7	*Sal*I subclone of pAVM1BamSm, in pBC SK$^+$	This study
pAVS8	*Sal*I subclone of pAVM1BamSm, in pBC SK$^+$	This study
pAVM2	*Eco*RI fragment with Tn*5* insert from M2, in pBSK$^+$	This study
pAVM2BamKm	*Bam*HI subclone of pAVM2, in pBC SK$^+$	This study
pAVM2BamSm	*Bam*HI subclone of pAVM2, in pBSK$^+$	This study
pAVM3	*Kpn*I fragment containing Tn*5*, from M1, in pBSK$^+$	This study
pAVE1	*Eco*RI subclone of pAVM3, in pBSK$^+$	This study
pAVP10	*Pst*I subclone of pAVM3, in pBSK$^+$	This study
pAVP10Ω14	pAVP10 with Ωspc insertion in *Eco*RI site	This study

Table 5. List of Plasmids Continued

Plasmid	Relevant Features	Reference/Source
pAV18PΩ	KpnI/PstI insert from pAVP10Ω14 in pUC18	This study
pAVJQΩ	Insert from pAV18PΩ in pJQ200SK^{+}	This study
pBAC11	pRK7813 cosmid clone from 248 containing bacteriocin genes	Oresnik *et al.* 1999
pBAC12	*Eco*RI subclone from 248 cosmid containing bacteriocin genes	Oresnik *et al.* 1999

plates using sterile toothpicks. Test strains were grown without antibiotics to avoid antibiotic carry-over except when antibiotics were necessary for plasmid maintenance. In this case, an indicator strain which was also antibiotic resistant was used: e.g. 248pRP4-4 was constructed for use as a tetracycline resistant (Tet^r) indicator strain.

Bacteriocin plates were incubated at 30 °C overnight or longer until clear halos were visible around the test strains.

3.2.2 Spot Test Method

The spot test method was developed to evaluate bacteriocin activity from supernatant fluid (SF). Indicator cultures were grown and diluted as in the stab method. Indicator overlays were made by adding 0.5 mL diluted indicator culture to 7 mL molten soft TY in a sterile test tube and pouring the mixture on a thin, dry TY plate. Indicator plates were generally spotted with test supernatant within two hours of pouring.

Test strains were typically grown up overnight in TY at 30 °C on a roller, and then subcultured (0.2-0.5 mL *A. tumefaciens* or *S. meliloti* into 100 mL PH or 0.250 mL into 50 mL PH; 0.5-1 mL of *R. leguminosarum* into 100 mL PH or 0.5 mL into 50 mL PH). For rapid, small scale preparations, TY cultures were grown for 1 to 2 days in test tubes at 30 °C on a roller before collecting the SF. For all small scale SF preparations, 1-1.5 mL of each broth culture was spun down at 14 000 rpm in a microcentrifuge. In all cases, an Eppendorf Centrifuge Model 5415C or a Biofuge A Centrifuge from Heraeus was used. The SF was filter sterilised using low protein-binding acrodiscs (pore size 0.20 μm or 0.45 μm), aliquotted to a sterile Eppendorf tube and stored at 4 °C or used immediately. For large scale preparations, cultures were generally centrifuged at 11 000 rpm (19 690 g) in a GSA rotor. In all cases, a Sorvall RC-5B Refrigerated Superspeed Centrifuge was used. The SF was then filtered using Nalgene filter units with pore size 0.22 μm, and used immediately or stored at 4°C, as in a similar protocol used by Hirsch

(1979).

For concentrated preparations 200 mL cultures were centrifuged at 11 000 rpm (19 690 g) for 1.5 hours in a GSA rotor using the high speed centrifuge, and then filtered through 0.22 µm Nalgene filter units. SF was concentrated approximately eight fold in an Amicon filtration device according to the manufacturer's instructions. YM30 filters were used in order to concentrate proteins 30 KDa or larger. Concentrates were filter sterilised with 0.45 µm acrodiscs and stored at 4 °C.

To test the effect of temperature on bacteriocin activity, prepared SF was incubated at a range of temperatures for 1 hour to 1 week prior to use in bacteriocin tests. To test the effect of HEPES, PBS, Tris buffer and a range of salt concentrations ($CaCl_2$), 0.5 mL of prepared SF was incubated with 0.5 mL of each test solution overnight to1week prior to use in bacteriocin tests.

10 uL aliquots of SF or concentrate were spotted onto solidified indicator plates. Plates were incubated upright at 30 °C overnight or longer until clear halos were visible.

3.2.3 Broth Culture Method

Bacteriocin activity in broth cultures was measured by growing the susceptible indicator strain in broth containing SF from the test strain.

Bacteriocin producers and controls were grown for four days with shaking at 28 °C in TY broth supplemented with 5 mM $CaCl_2$. Cultures were spun down at 11 000-12 000 rpm (19 690-23 430 g) for 30 minutes. Some of the SF was filtered though 0.45 µm acrodiscs. Filtered and unfiltered SF was stored at -70 °C for several weeks. SF was then thawed overnight at 4 °C and for five minutes at 37 °C. Approximately 20 mL SF was added to 25 mL of TY broth for bacteriocin testing (5 mM $CaCl_2$). The broth/SF was inoculated with 0.5 mL of fresh 336 broth culture. The test cultures were then incubated with shaking at 28 °C. The OD600nm was checked over the course of 150 hours, and at

the end of this period a plate count was made. Similar trials were also carried out using a Klett photometer.

This experiment was repeated two times with chloroform treated SF, as follows. Cultures were grown in TY for bacteriocin testing for three days at 28°C with shaking. Cultures were then spun down in an ultracentrifuge at 11 000 rpm (19 690 g) for 30 minutes. 5% vol/vol chloroform was added to the SF (approximately 90 mL) in an extraction funnel. Treated SF was allowed to sit in open containers in the fumehood for 3 to 10 hours to allow residual chloroform to settle or evaporate. SF was then filtered through 0.45 µm acrodiscs. 20 mL SF was added to 20 mL TY for bacteriocin testing, and the mixture was inoculated with 0.5 mL of an overnight 336 broth culture. Test cultures were incubated with shaking at 28°C, and the OD600nm was monitored over a period of 145 hours.

3.3 Analysis of *Rhizobium* Plasmids

3.3.1 Modified Eckhardt Protocol

Due to the large size of rhizobial plasmids, traditional methods for plasmid isolation (i.e. alkaline lysis) are not useful in rhizobia, and therefore typical restriction analysis of plasmid DNA cannot be employed to study these plasmids. In a technique developed by Eckhardt (1978), rhizobial plasmid profiles can be obtained by lysing the cells in the wells of an agarose gel, and allowing the plasmid DNA to migrate through the gel in covalently closed circular form.

Rhizobial plasmids and RP4-4 were therefore visualised on modified horizontal Eckhardt gels (Hynes *et al.* 1985; Hynes *et al.* 1986). Cultures were grown overnight in PH medium. Then, 0.1 mL to 1.0 mL of overnight cultures were added to Eppendorf tubes on ice; 0.5 mL 0.3 % cold Sarkosyl in tris-borate buffer was added to each sample and the tubes mixed by inversion. Samples were then spun for five minutes at 14 000

rpm in a microcentrifuge and the supernatant fluid removed with a broken needle attached to a syringe. In order to remove as much of the supernatant fluid as possible, samples were spun for an additional two minutes as needed. *R. leguminosarum* bv. viciae 306 and 309 were left on ice in the cold room for three hours. Samples were then resuspended in 20 µL of lysis solution containing approximately 0.1 mg of lysozyme, which was removed from the container using a sterile needle, and resuspended in 0.5 to 1 mL E1 (10% sucrose, 10 µg/mL RNase in tris-borate buffer, autoclaved and stored at 4 °C) and immediately loaded into the wells of an SDS-agarose gel (0.6 g agarose, 80 mL tris-borate buffer, 8 mL 10% SDS, or half of these amounts). All gels were run at room temperature. Gels were run at 5-8 volts for up to 1 hour to allow for complete lysis of the cells. The gels were typically run for a further 4-6 hours at 76 volts, and stained overnight in ethidium bromide (approximately 20 µg/mL). DNA was visualised under a UV light and photographed.

3.3.2 Mobilisation and Curing of Plasmids from *Rhizobium*

The function of a given rhizobial plasmid may be ascertained by moving the plasmid in question to another strain, or by plasmid-curing. In order to mobilise non self-transmissible plasmids, plasmids tagged with Tn-*mob* can be mobilised into other strains using RP4 as a helper plasmid (Simon 1984). Tn-*mob* contains the origin of transfer from RP4, a broad host-range plasmid, cloned into a Tn5 derivative. The transposon also carries Gentamicin resistance for selection purposes. Tn-*mob-sac*, a derivative of Tn-*mob*, also carries the *sacB* gene, which is lethal when expressed in Gram negative bacteria (Hynes *et al.* 1989; Quandt and Hynes 1993). 306SM was tagged with Tn-*mob-sac* and a number of isolates was obtained (Twelker and Hynes, unpublished). By growing a number of these isolates at 45 °C on media containing sucrose, mutants cured of pRle306b were obtained (Hynes, unpublished).

For mobilisation of the 306 plasmids into UBAPF2, Rm1021 and AK631, 306 strains were first mated with *E. coli* J53 RP4-4 and Tet^r clones selected(Hedges and Jacob 1974). The resulting 306/pRP4-4 exconjugates were then mated with the recipient strains. Most other matings were carried out as triparental matings.

3.3.2.1 Method for Conjugation

Strains were grown as broth cultures until reaching log phase. Aliquots of donor and recipient strains, and the helper strain in triparental matings, were added to the same Eppendorf tube and spun down at 14 000 rpm. Pellets were resuspended in 50 µL broth media and spotted on TY plates. Mating spots were incubated at 30 °C overnight. Spots were then scraped up and resuspended in broth media or sterile water. The cells were further diluted in broth or sterile water and aliquots of the dilution series plated out on selective media. Plates were incubated at a permissive growth temperature for the recipient strain for up to five days.

Helper strains used in this study included RP4-4 which was used to mobilise Tn-*mob-sac* tagged 306 plasmids, and MT616 pRK600 which was used to mobilise all other plasmids into and out of *Rhizobium* and *E. coli* (Finan *et al.* 1986; Hedges and Jacob 1974).

3.4 Genetic Analysis

3.4.1 Construction and Screening of the Tn*5* Mutants

Tn*5* mutants were constructed by using the suicide vector, pRK602, which is unable to replicate in *Rhizobium*, to deliver Tn*5* to the recipient strain, 306SM. 306SM was mated with the donor, *E. coli* strain MM294a/pRK602. Mating spots were resuspended in water and a dilution series was made and plated out on a selective medium, TY/neomycin (Nm)/streptomycin (Sm). Single colonies were isolated from the

10^{0} and 10^{-2} dilution plates, repatched on selective medium and plain TY plates, and incubated for two to three days. Each repatched isolate was then tested for bacteriocin activity against 248 and VF39. Isolates were toothpicked from plain TY plates and stabbed into indicator plates of 248 or VF39. Plates were incubated at 30 °C over 48 hours and then scored for the presence/absence and quality of halos.

3.4.2 Small-Scale Isolation of Genomic DNA from *Rhizobium*

Overnight 4 mL TY cultures were spun down at 14 000 rpm in a microcentrifuge and the SF removed. Pellets were resuspended each in 1 mL TE 50:20 (Tris-HCl 50 mM, pH8; ETDA 20 mM). The cells were spun down again, the SF removed and the pellets resuspended each in 0.4 mL TE 50:20. The cells were then lysed by gently mixing each aliquot with 50 µL predigested pronase (5 mg/mL in TE 50:20 incubated at 37 °C for 1 hour prior to use), followed by gently mixing with 50 µL SDS (10% in TE 50:20). The samples were incubated at 37 °C for 1 hour, with gentle agitation every 15 minutes. The cleared lysate was then passed through a clean syringe and sterilised needle to fragment the DNA. This step was repeated two more times. To extract lipids and other cellular debris from the DNA, 0.5 mL phenol:chloroform 1:1 was added per sample and the tubes vortexed thoroughly. Each sample was spun for 5 to 20 minutes, the top layer transferred to a clean Eppendorf tube, and 50-100 µL TE 50:20 added to maintain the sample volume. Extraction with phenol:chloroform 1:1 was repeated two times and followed by extraction with 0.5 mL chloroform. DNA was then precipitated with 15 µL NaCl 5M and 0.8 mL cold 95% ETOH. Tubes were left at -70 °C for 20 minutes and then spun down in a microcentrifuge at 4 °C. ETOH and salt were poured off and the DNA was washed with cold 75% ETOH, centrifuged for 1 minute at 4 °C, and the pellets dried. Samples were resuspended in 50 µL TE 10:1 (Tris-HCl 10 mM, EDTA 1 mM) plus 1 µL RNase 10 mg/mL, or stored at room temperature for future use.

3.4.3 Large Scale Isolation of DNA from *Rhizobium*

Strains were grown up in 100 mL TY for 48 to 60 hours at 28 °C with shaking. Log phase cultures were spun down for 15 minutes at 10 000 rpm (16 270 g) in the high speed centrifuge and the SF removed. Pellets were washed by resuspending in 10 mL TE-1 (Tris-HCl 20 mM, pH 8; EDTA 20 mM, pH 8; NaCl 150 mM). Cells were centrifuged as above, the SF removed and the pellets frozen at –70 °C. Cell pellets were checked for purity by streaking them out on TY plates. Frozen pellets were allowed to thaw slightly, and then were suspended in 5 mL TE-2 (Tris-HCl 20 mM, pH 8; EDTA 20 mM, pH 8; Sarkosyl 1%) and centrifuged at 10 000 rpm (16 270 g) for 15 minutes. SF was poured off and each pellet was resuspended in 10 mL TE-3 (Tris-HCl 20 mM, pH8; EDTA 20 mM, pH 8). To each sample, 1 mL lysozyme (0.5 mg/mL in sdH_2O) was added and the cells allowed to lyse for 15 minutes at 37 °C. Predigested pronase and Sarkosyl were mixed for a final concentration of 0.5 mg/mL pronase and 1% Sarkosyl in sdH_2O, and 10 mL of the mixture was added to each DNA sample. Cells were allowed to lyse for a further 80 minutes at 37 °C with gentle rocking. Cell wall components were extracted by adding 4 mL chloroform:phenol 1:1 per tube, gently rocking the tubes several times, then spinning the samples for 10 minutes at 6000 rpm (4302 g) in an SS34 rotor in the high speed centrifuge. The top layer and interphase of each sample was poured off into a clean centrifuge tube and two more extractions performed, using only 2 mL chloroform per sample each time. (DNA was stored overnight at 4 °C between the last two extractions). After the final extraction only the top layer was saved. DNA was precipitated in glass centrifuge tubes with two parts cold 95% ETOH containing 10 % ammonium acetate 3 M. Samples were centrifuged at 6000 rpm (4302 g) for 10 minutes and the ETOH and buffer poured off. Each centrifuge tube was rinsed with 1 mL TE-4 (Tris-HCl 20 mM, pH 8; EDTA 1 mM, pH 8) to dissolve the DNA, which was stored at 4 °C (Miller 1972; Sambrook *et al.* 1989).

3.4.4 Restriction Analysis

Restriction enzymes were purchased from Amersham Pharmacia Biotech and GIBCO-BRL, Life Technologies, and used according to the manufacturer's instructions, generally with GIBCO-BRL React buffers.

Samples were run on 0.6 % to 1 % agarose gels (typically 0.7% for total DNA and 0.8 % for plasmid DNA) made in tris-borate buffer (see Appendix A). Gels were run horizontally and at room temperature, overnight at about 12 V, or for 1 or more hours at 100 V or less.

Gels were stained in ethidium bromide (approximately 20 µg/mL) and the DNA visualised and photographed under a UV light.

3.4.5 Southern Blots

Agarose gels were destained in dH_2O and then soaked for 15 minutes in HCl 0.25 N to denature the DNA. Gels were then neutralised in two 15 minute changes of NaOH 0.5 M/ NaCl 0.15 M. Gels were blotted with nitrocellulose membranes. The membranes were cut to fit the gel dimensions, and then lain on top of the gels. Pieces of Whatman filter paper, also cut to fit the gel dimensions, were soaked in transfer buffer (NaOH 0.4 M/ NaCl 0.6 M). For each gel, three pieces of filter paper were placed on a flat, plastic tray, and then placed underneath the gel, while four pieces of filter paper were placed over top of the membrane. Each sandwiched gel was compressed with stack of paper towels and a weight. Transfer was allowed to occur overnight at room temperature. Blotted membranes were marked, washed in 5xSSC for 15 minutes, air dried briefly, then baked in a vacuum oven at 80°C for 30 minutes, as suggested by the manufacturer (Boehringer-Mannheim, Roche Diagnostics). Membranes were sealed in saran wrap until detection was carried out.

3.4.5.1 Detection Procedure for Southern Blots

The protocol was adapted from that given for the use of positively charge nylon membranes made by Boehringer-Mannheim, Roche Diagnostics. Each baked membrane was washed in 5XSSC for 10 minutes, then pre-hybridised at 65-68 °C with hybridisation buffer in a hybridisation bottle for 1 to 2 hours. The probe was boiled for 10 minutes, then cooled at -80 °C for three minutes. The cooled probed was poured into the hybridisation tube and allowed to hybridise to the membrane at the pre-hybridisation temperature overnight in the hybridisation oven.

Stringency washes of the membrane were performed as follows: 1. Two 15 minute washes with 50 mL 2XSSC/0.1% SDS at room temperature; 2. Two 15 minute washes with 50 mL 0.1XSSC/0.1% SDS at the hybridisation temperature.

Chemiluminescent detection of the bound probe using CSPD was carried out according to the manufacturer's instructions (Boehringer-Mannheim, Roche Diagnostics). Membranes were exposed to CSPD in a sealed plastic bag for up to 1 minute at room temperature before the excess was removed. X-ray film was exposed to the membrane in a light-tight cartridge at 37 °C, typically for 45 minutes, but with exposure times ranging from 20 minutes to 3 hours. Exposed X-ray film was developed in developer (90 seconds), washed in water, fixed in fixative (90 seconds) and water washed before air drying.

Used membranes were stripped with two 15 minute washes of NaOH 2 N plus SDS 1% in dH_2O, and wrapped in saran wrap for storage.

3.4.5.2 Construction of Tn*5* Probe for Use in Southern Detection

The procedure for labeling the inner *Bgl*II fragment of Tn*5* was simplified from the one given by Boehringer-Mannheim, Roche Diagnostics for use of the nonradioactive DNA Labeling and Detection Kit. The kit works by random primed incorporation of

digoxigenin (Dig) – labeled dUTP into the probe DNA. Following Southern hybridisation with the labeled probe, anti-Dig antibody-alkaline phosphatase conjugate is allowed to bind to the probe. Subsequent enzymatic dephosphorylation of CSPD produces a metastable anion, which decomposes to emit light at a wavelength sufficient to expose X-ray film.

Plasmid DNA was first isolated from *E. coli* S17-1 containing pSUPGm::Tn*5*B20 using the Promega Wizard kit (Simon *et al.* 1989). 35 µL of *Bgl*II restricted Tn*5*-B20 was run on a 1 % agarose gel across two lanes. The inner *Bgl*II fragment of Tn*5* (2.66 kb) was gel isolated using the Prep-a-gene kit, according to Bio Rad's instructions. Gel isolated DNA was incubated in a boiling water bath for 10 minutes, then chilled quickly at -80 °C. The following reaction mixture was set up in an Eppendorf tube:

25 µL DNA (at 50 ng/µL as estimated by comparison to λ marker DNA on restriction gels)

2 µL hexanucleotide mixture (10X concentration)

2 µL DIG-dNTP labeling mixture (10X concentration with dATP, 1 mM; dCTP, 1 mM; dGTP, 1 mM; dTTP, 0.65 mM; Dig-UTP, 0.35 mM)

1 µL Klenow fragment of polymerase (2 U/µL).

The reaction mixture was incubated at 37 °C for about 23 hours. The probe was added to approximately 14 mL hybridisation buffer, boiled for 10 minutes, cooled quickly at -80 °C, then used for Southern detection and subsequently stored at -20 °C.

3.4.6 Cloning of the Regions Flanking the Tn*5* Mutations

pBSK$^+$ and total DNA were cut with *Eco*RI or *Kpn*I. The DNA was then run on agarose gels to check for complete digestion and to determine the relative concentrations of DNA compared to the *Hind*III digested λ marker and to each other. Cut DNA was then incubated in a 70 °C water bath for 10 minutes to inactivate the enzyme. The DNA

was allowed to cool to room temperature before ligation or storage. The DNA was not precipitated or processed further before ligation. The DNA was mixed in a 5:1 or 3:1 ratio of genomic DNA to vector DNA, ligated together, and transformed into competent cells as described in the following sections.

3.4.6.1 Ligation Reactions

Ligation reactions were carried out according to the manufacturer's instructions (GIBCO-BRL, Life Technologies). Reactions were set up as follows:

1 µL T4 ligase

3 to 5 µL total DNA to 1 µL vector (at DNA concentrations of 50 ng/µL, as estimated by comparisons to λ marker DNA in restriction gels)

4 µL T4 ligase buffer

volume adjusted to 20 µL with sterile dH_2O

For all cloning, DH5α was transformed with 10 µL of each ligation, or 1 µL uncut vector DNA as a control. Transformants were selected by growth on X-gal/ampicillin (Amp) or chloramphenicol (Cm) plates, or Amp, Cm or kanamycin (Kan) plates.

3.4.6.2 Preparation of Competent Cells

50 or 100 mL *E. coli* DH5α cultures were grown up for 18 hours in LB broth and then subcultured and grown for approximately 2 hours (Sambrook *et al.* 1989). Cultures were placed in chilled centrifuge tubes and centrifuged at 6000 rpm (4302 g) at 4 °C for 10 minutes. Supernatant fluid was discarded and each cell pellet resuspended in 20 mL of 0.1 M $MgCl_2$. Cells were spun at 6000 rpm (4302 g) at 4 °C for 10 minutes. Supernatant fluid was discarded and each cell pellet resuspended in 0.1 M $CaCl_2$. Cells were incubated on ice for 30 minutes or more, then spun at 6000 rpm (4302 g) at 4 °C for 10 minutes. Supernatant fluid was discarded, each cell pellet resuspended in about 2 mL

0.1 M $CaCl_2$/ 14 % glycerol, and the cells aliquotted (200-300 µL) into chilled Eppendorf tubes. Cells were frozen and stored at -80 °C.

3.4.6.3 Method for Transformation of Competent Cells

The procedure used is described by the manufacturer of Max Efficiency DH10β Competent Cells (GIBCO-BRL, Life Technologies). Competent DH5α cells (as described above) were used for transformation. Changes to the protocol were as follows: following heat shock and recovery on ice, each tube of cells and DNA was enriched with 900 uL of two times LB (see Appendix A) and then incubated at 37 °C for 1 hour or more. Cells were then plated out on selective media and the plates incubated overnight at 37 ° C.

3.4.6.4 Alkaline Lysis Mini-Plasmid Preparations

One mL of an overnight broth culture was spun down at 14 000 rpm in the microcentrifuge, the supernatant fluid removed, and the pellet resuspended in 100 µL solution I (glucose 50 mM, Tris 25 mM, EDTA 10 mM, pH8) (Sambrook *et al.* 1989). Cells were lysed by adding 200 µL solution II (NaOH 0.2 N, SDS 1% in sdH_2O) and inverting the tube several times. To increase the pH, 250 µL solution III (potassium acetate 5M, glacial acetic acid 11.5 % in sdH_2O) was added and the tube inverted several times. Tubes were spun for 20 minutes or longer at 14 000 rpm and the supernatant kept. 200 µL of chloroform was added to the DNA and the samples spun for 1 minute at 14 000 rpm. The top layer was pipetted out. One mL cold 95% ETOH was added to the DNA at which point the samples were typically spun for 30 minutes at 4 °C. Alternately, samples were stored at -20 °C and later spun down at room temperature. Precipitated DNA was washed with 70% ETOH followed by 95% ETOH, and then dried in the vacuum oven. Pellets were either stored for later use or resuspended in 25 µL

sdH_2O, generally by dissolving at 65 °C for 10 minutes. Resuspended plasmid DNA was stored at 4 °C.

3.4.6.5 Subcloning and Sequencing from the Tn5 Clones

Nucleotide sequencing was carried out by subcloning and primer walking. Sequencing reactions were carried out by the University of Calgary Core DNA Facilities by using dye terminators and detection with an ABI automated sequencer.

Sequencing of the pAVM1, pAVM2 and pAVM3 clones was performed using the T3 and T7 primers of $pBSK^+$. The ends of the subclones were also sequenced from T7 and T3 from $pBSK^+$ or pBC SK^+, and the regions flanking Tn*5* were sequenced from IS*50* primers from IS*50* in the transposon (see results section).

To create subclones, pAVM1 and pAVM2 were digested with *Bam*HI and the fragments self-ligated or ligated into pBC SK^+ (see results section, Figures 13 and 14). Selection for transformants was made on LB-Cm/X-Gal plates or LB-Amp plates. Restriction analysis was used to screen the transformants for potential positive clones. One of the *Bam*HI subclones (pAVM1BamSm) was restricted with *Sal*I and *Cla*I and the fragments self-ligated or ligated into pBC SK^+. Selection for transformants was made on LB-Amp/X-Gal plates or LB-Cm/X-Gal plates. pAVM3 was digested with *Eco*RI, and re-ligated, and selection for transformants was made on LB-Amp plates (see results section, Figure 15).

Sequence data were analysed with DNASIS (Hitachi Software Engineering Co., San Bruno, Calif.). Genbank searches were done with the BLASTX program (Altschul *et al.* 1990).

3.5 Construction of Transporter Mutants

pAVM3, the clone containing the *Kpn*I insert from M1, was digested with *Pst*I

and religated (see Figure 21 in results section). The Ωspectinomycin (Spc) cassette from pHP45Ωspc was introduced into the unique *Eco*RI site in the subclone (Prentki and Krisch 1984). The genetic region flanking the Ωspc cassette was removed with *Kpn*I and *Pst*I and cloned into those sites in pUC18 in order to introduce a n *Sst*I site next to the insert. The insert was then cut out with *Sst*I and *Pst*I. and cloned into those sites in the gene replacement vector, pJQ200SK$^+$ (Quandt and Hynes 1993). The resulting clone was mated with MT616pRK600 and 306DM or 306SM in order to introduce the cassette into *Rhizobium* using the gene replacement vector. 306SM mutants were selected on TY-Sm/Spc/sucrose 5%. 306DM mutants were selected on VMM-Spc/sucrose 5%. Putative mutants were restreaked on TY-gentamicin (Gm) and checked for Gm sensitivity to ensure that double-crossovers had taken place and that the vector was probably not contained in the clones.

3.6 Transfer of the Tn*5* Mutation from a Fix$^-$ Strain to Fix$^+$ Strain

pAVM1 was created by cloning out the genetic region flanking the Tn*5* insertion in M1 as an *Eco*RI insert. M1 is Fix$^-$, as is its parent strain 306SM. This *Eco*RI insert was then cloned out of pBSK$^+$ and inserted into the *Eco*RI site in pRK7813 to produce a Tet^r clone, pAVRKM1 (see Figure 22, results section) (Jones and Gutterson 1987). After every transformation, potential positive clones were checked by restriction analysis to ensure that the inserts were the correct size. The insert from pAVRKM1was then cloned into the *Eco*RI site in pUC1819 in order to introduce flanking *Xba*I sites, since this site was also available in the gene replacement vector. Transformants were selected for on LB-Amp/ plates (Schweizer 1993). The insert was cloned out from flanking *Xba*I sites in the vector, and introduced into the *Xba*I site in pJQ200SK$^+$. Resulting clones were mated with 306DM (Fix$^+$) and a strain carrying the helper plasmid, MT616/pRK600. Mutants were selected on VMM-Nm/sucrose (5%), and cross-checked for Gm sensitivity to

ensure that double-crossovers had taken place and that the vector was probably not contained by the 306DM::Tn*5* clones. Clones were analysed by Southern hybridisation.

3.7 Competition Experiments

3.7.1 Preliminary Competition Experiments

Strains were grown up for 36 hours in TY broth. Optical density readings were taken, and an equivalent amount of each culture was diluted in sterile water for an initial dilution. Plate counts were conducted at this time to confirm that the initial cell numbers used were the same. Equal amounts of cells were used to co-inoculate 5 mL TY broth, and the cultures grown for approximately 48 hours on the roller at 30 °C. The initial plate counts were taken into account in the analysis of the data, in order to adjust for discrepancies in the inoculation ratios. Following incubation, cultures were plated out from a dilution series onto TY plates. Isolated colonies were patched onto TY and selective media in order to determine the ratio of each strain in the co-cultures.

Results were analysed using the chi-square (χ^2) test to evaluate the significance of differences between competing strains, the null hypothesis being that there would be no significant difference. The significance of the difference between the abilities of the 306 strains to compete with other strains was also determined using the χ^2 test. In these cases, the observed value for 306SM was used as the expected value for the other 306 strains. The formula used is given below:

$\chi^2 = \Sigma (O-E)^2/E$, where O is the observed value, and E is the expected value, given the inoculation ratio.

3.7.2 Plant Competition Experiments

The method used for the plant experiments is described by Hynes and O'Connell 1990). Trapper peas were surface sterilised with 95% ETOH for five minutes followed

by 100 mL 20% chlorox bleach in sterile water for five minutes. Seeds were then rinsed in 10 changes of sterile water. Vetch seeds were surface sterilised with two five minute washes with 95% ETOH, 10 minutes in 20% chlorox bleach, 1 minute in 95% ETOH, and a further 10 minutes in fresh 95% ETOH. Seeds were then rinsed in 10 changes of sterile water.

All seeds were allowed to germinate on water agar plates at room temperature in the dark for 72 to 96 hours. Seedlings were planted in Magenta jars containing sterile plant growth medium and vermiculite and the jar lids replaced. The pots were then incubated in a growth chamber, and after three to five days, the seedlings were inoculated. The bacterial cultures were grown to log phase over 36 hours. In order to ensure that the ratios of the inocula were equal, optical density readings were taken of the cultures, and an equivalent amount of each culture was diluted in sterile water (to about 10^{-2}) before being used to inoculate the pots. 3 to 5 mL of diluted culture was used to inoculate each pot. Plate counts were also conducted to confirm that the initial cell numbers used were equivalent. Any differences in the inocula ratios were accounted for in the final analysis of the data. Jar lids were removed following inoculation, generally after one day. Plants were grown for 4 to 5 weeks in a growth chamber and watered only with sterile water.

The nodules were then harvested over a period of 6 days. Nodules were surface sterilised for 1 minute in 20% chlorox bleach and washed about 6 times in sterile water. The nodules were crushed in sterile Eppendorf tubes containing 50 µL aliquots of sterile water. Crushed nodules were spotted on TY plates and selective media (Josey *et al.* 1979). In one competition trial, spots growing on the TY plates were also stab inoculated into 248 indicator plates to check for the presence of bacteriocin activity, in order to distinguish the competing strains from one another. χ^2 analysis was performed as described above, on the data pooled from 3 to 5 pots. Systat was also used to evaluate the variance of the means between different pots of plants.

4.0 Results: *Rhizobium leguminosarum* bv. viciae strain 306 Produces Three Unique Bacteriocins

4.1 306 Inhibits at Least Eight Closely Related Strains

Bacteriocin production in *Rhizobium* is wide-spread (Hirsch 1979; Lotz and Mayer 1972; Roslycky 1967; Schwinghamer and Belkengren 1968; Schwinghamer 1971; Schwinghamer 1975). We were interested in studying a previously uncharacterised bacteriocin from *Rhizobium*. Therefore, the cross-resistances between various strains of *Rhizobium leguminosarum*, *Rhizobium etli*, *Sinorhizobium meliloti* and *Agrobacterium* were tested in order to pin-point one strain with a significant range of bacteriocin activity (Table 6). Of the strains tested, 306 inhibited 8 indicators, which was a larger number of strains than most other strains tested (Table 6). The bv. viciae strain 309 exhibited a similar range of bacteriocin activity to 306. This was expected, since 306 and 309 have very similar plasmid profiles and are likely extremely closely related (Hirsch *et al.* 1980; Hynes, personal communication). Bacteriocin production by 306 was thus chosen as the focus of further study.

Small bacteriocins are known to produce wide zones of inhibition on indicator plates, while medium bacteriocins make smaller zones of inhibition on indicator plates (Hirsch 1979). Also, small bacteriocins are not known to inhibit other small bacteriocin producers (Hirsch 1979). 306 is known to be a medium bacteriocin producer (Hirsch 1979). The quality and size of the zones of inhibition produced by and against 306 were therefore closely examined. It was observed that zones of inhibition produced by 306 on indicator plates were extremely clear compared to zones of inhibition produced by small bacteriocin producers such as 336, 3841 and VF39. Zones of inhibition produced by 306 stabs on 248 indicator plates were of similar size and quality as zones of inhibition produced by 248 on 336 indicator plates. These halos were small and clear (approximately 1 to 8 mm in diameter) compared to the zones of inhibition produced by

Table 6. Cross-Resistance of *Rhizobium, Sinorhizobium and Agrobacterium*

Strains[abc]

Indicators	**Test Strains**

	Brazil 5	CFN42	F8	Viking1	1-3	W14-2	162Y10	GF160	VF39	248	306*	309	336	CFN299	CIAT 899	**AK631**	**cc2013**	**ML1**	**ML2**	**ML3**	**ML4**	**ML5**	**RCR2012**	**Rm1021**	**102F34**	**102FS1**	**104A14**	**Br816**	*ChAg4*	*Kag3*
Brazil 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CFN42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F8	-	-	-	-	-	?	?	s	s	?	?	s	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Viking1	-	-	-	x	-	x	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-3	-	-	-	-	-	x	?	+	?	+	+	?	-	-	x	x	x	x	x	x	x	x	x	x	x	-	-	-	-
W14-2	-	-	-	-	-	-	?	?	?	+	?	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
162Y10	-	-	-	x	-	-	-	-	-	+	+	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GF160	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VF39	-	-	-	?	?	?	+	+	-	+	+	+	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	?	-
248	-	-	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	?	?	-
306*	-	-	?	?	?	?	-	?	?	-	-	-	?	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
309	-	-	?	?	+	?	?	+	-	-	-	-	+	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
336	-	-	-	-	x	?	+	?	-	+	+	+	-	-	-	-	?	-	-	-	?	-	-	s	-	-	-	-	+
CFN299	-	-	-	-	?	-	?	?	s	s	?	?	s	-	-	-	-	-	-	-	-	-	-	?	-	-	-	-	-
CIAT 899	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	-	-	?	-	-	-	-	-
AK631	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cc2013	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ML1	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-
ML2	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	s	-	-	-	-	-
ML3	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ML4	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ML5	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	?	?	-	-	s	+	-	-	+	?	-	-	-	-
RCR2012	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Rm1021	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
102F34	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	?	-	-	-	-	-	-	-	?	-	-	-	-	-
102FS1	-	-	-	-	x	x	x	x	-	-	x	x	-	-	-	-	-	-	-	-	-	-	-	?	-	-	-	-	-
104A14	-	-	-	-	x	x	x	x	-	?	x	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Br816	-	-	-	-	-	-	x	-	-	-	-	?	-	-	-	-	-	-	-	-	?	-	-	+	-	-	-	-	-
ChAg4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kag3	-	-	-	-	x	-	x	?	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ , halo; -, no halo; s, small halo; ? results unclear or conflicting in different tests; x not tested

[a] Stab tests were used to test for bacteriocin production/resistance. [b] *Rhizobium* strains are in regular type and grouped as *R. etli, R. leguminosarum* bvs. trifolii and viciae, and R. tropici. *Sinorhizobium* strains are in bold, and *Agrobacterium* strains are italicised. [c] Although 306SM has the same plasmid profile as the wt 306 strains, 306SM is not clearly inhibited by 336, VF39 or Viking 1, whilst 306wt is inhibited.

small producers 336 or 3841 on 248 indicator plates (approximately 1 cm or larger in diameter). In stab tests and spot tests using fresh supernatant fluid (SF), 306 produced larger, clearer zones of inhibition on 336 indicator plates than on 248 indicator plates or 162Y10 indicator plates. Zones of inhibition on 336 indicator plates were quite large and extremely clear (over 1 cm in diameter). The zones of inhibition produced by the 306 strains against 248 and 162Y10 were characteristic of halos resulting from medium bacteriocin production, while zones of inhibition produced by the 306 strains against 336 were uncharacteristically large.

Some differences were noted in the quality of the halos made against the 306 strains used in the study (306SM, 306wt, 306DM). Zones of inhibition produced by small bacteriocin producers 336 and 3841 on 306 indicator plates were large and fuzzy (not clear), as if there was a very low level of background growth on the indicator plates. 336 stabs produced fuzzy but clearer halos on 306wt indicator plates than on 306SM plates, and 336 halo production on 306SM plates (which did not contain antibiotics) was inconsistent and often difficult to read. 3841 stabs produced large, fuzzy zones of inhibition against all of the 306 strains, with clearer zones of inhibition on 306wt and 306DM indicator plates than on 306SM indicator plates. VF39 also produced fuzzy but clearer zones of inhibition against 306wt than 306SM.

4.2 Bacteriocin Activity in 306 is Plasmid Mediated

Bacteriocin activity in *Rhizobium* is sometimes plasmid mediated (Hirsch 1979). The 306 plasmid pRle306b is known to confer bacteriocin activity to other strains of *Rhizobium leguminosarum* (Hirsch 1979). It was of interest to determine whether or not each of the other plasmids from 306 could mediate bacteriocin activity in other rhizobial strains. A variety of 306SM strains was available which had been mutagenised with Tn-*mob-sac* (Hynes *et al.* 1989; Twelker and Hynes, unpublished). The mutants showed

normal growth on TY plates. Each mutant harboured the Tn-*mob-sac* in one of its plasmids (pRle306a through e), which could therefore be mobilised into other strains using the helper plasmid RP4-4.

Each 306SM plasmid was mobilised into *A. tumefaciens* UBAPF2, and *S. meliloti* strains AK631 and Rm1021. An attempt was also made to mobilise these plasmids into *R. leguminosarum* strains 8401, 4292 and B157, but transfer was largely unsuccessful, possibly due to incompatibility with genetically similar resident plasmids. Transfer of the 306 plasmids was confirmed by the appearance of appropriately sized new plasmids in Eckhardt gels of the recipient strains. These plasmids banded at the same level as the 306 plasmids, in the 306 control lanes. pRle306a and pRle306b, which are very close in size and band as a doublet in Eckhardt gels, were distinguished based on the ability of pRle306b to transfer bacteriocin activity against 336 to recipient strains. Furthermore, since pRle306b is a self-transmissible plasmid, the transfer frequencies for a or b Tn-*mob-sac* tagged plasmids were determined. In the absence of a helper strain, tagged isolate 306SM::Tn-*mob-sac*-55 did not transfer gentamicin resistance to UBAPF2 at a detectable frequency, while tagged isolates 306SM::Tn*mob-sac*-12, 32, 38, 59, 73, 94, 95 and 30 transferred gentamicin resistance to UBAPF2 at an extremely high frequency, indicating that the tagged plasmids in isolates 12, 32, 38, 59, 73, 94, 95 and 30 were self-transmissible.

Each plasmid was then tested for bacteriocin activity. Bacteriocin activity from Rm1021 carrying each of the 306 plasmids was difficult to discern: stab tests were unclear and the negative control (Rm1021) was positive in many cases, while no bacteriocin activity was detectable from any of the clones in the SF assays. Bacteriocin activity from AK631/pRle306c was detected in stab plates but not SF assays. UBAPF2/pRle306b had activity against 336 in both stab and SF tests (Table 7). pRle306b conferred the same pattern of activity to UBAPF2 as seen from 248.

Table 7. Bacteriocin Production by 306 is Plasmid Mediated

Indicator Strains	Strains Tested for Production							
	248	306	UBAPF2	UBAPF2 pRle306a	UBAPF2 pRle306b	UBAPF2 pRle306c	UBAPF2 pRle306d	UBAPF2 pRle306e
248	-	+	-	-	-	+	-	-
336	+	+	-	-	+	-	-	-
VF39	+	+	-	-	+	W	-	-
3841	?	+	-	-	?	-	-	-
162Y10	-	+	-	-	-	-	-	-

- no inhibition, + inhibition, ? inhibition inconsistent, W weak inhibition

Stab tests and SF tests were used to detect bacteriocin activity.

UBAPF2/pRle306c had activity against 248 in both stab and SF assays (Table 7).

4.3 pRle306b and pRle306c do not Account for All Bacteriocin Activity from 306SM

The 306 plasmids pRle306a, d and e were not linked to bacteriocin activity. However, the full range of activity expressed by 306SM (e.g. anti-162Y10 activity) was not expressed by any of the 306 plasmids in UBAPF2 or AK631 (Table 7). It was also noted that zones of inhibition produced by 306SM on 336 indicator plates were somewhat clearer than zones produced by UBAPF2/pRle306b. UBAPF2/pRle306c showed no significant activity against 336.

306SM isolates cured of pRle306b (provided by Dr. Hynes) were tested for bacteriocin activity against several indicator strains (Table 8). The cured strains showed a range of activity comparable to 306SM, in that they inhibited the same strains as the parent strain inhibited.

Taken together, these observations indicated that the bacteriocin activity mediated by pRle306b and pRle306c did not account for all of the bacteriocin activity of 306SM, and that the additional bacteriocin activity was not mediated by one of the other plasmids alone.

4.4 Bacteriocins Encoded on pRle306b and pRle306c have Different Physical Properties

The medium bacteriocins examined by Hirsch were found to be heat labile at 85 °C, insensitive to proteolytic enzymes and incapable of diffusing through cellophane (1979). In addition, chloroform treated SF from 248 retained bacteriocin activity when stored at 4 °C for several weeks (Hirsch 1979). The physical stability of the bacteriocins of strain 306 was examined and compared to previous findings. To this end, SF from 306SM, UBAPF2/pRle306b and UBAPF2/pRle306c was analysed for bacteriocin

Table 8 . The Effect of Curing pRle306b on Bacteriocin Activity

Indictator Strains	306 Strains Tested for Production			
	-pRle306b12.3	-pRle306b12.4	-pRle306b30.2	-pRle306b30.3
248	+	+	+	+
336	+	+	+	+
VF39	?	+	?	+
3841	+	+	+	+
162Y10	+	+	+	+

+ inhibition, ? inconsistent inhibition

SF assays were used to detect bacteriocin activity.

activity after being subjected to various treatments (Tables 9 and 11).

In one trial, SF was incubated for 1 hour at various temperatures (Table 9). Anti-336 activity was retained by 306SM and UBAPF2/pRle306b samples incubated at 45 °C and lower, but not at 65 °C. Anti-248 activity was retained by 306SM samples incubated at 37 °C and lower, but not at 45 °C. In a separate trial, UBAPF2/pRle306c retained anti- when frozen or refrigerated (4 °C) for several weeks. 248 SF was also shown to 248 activity after incubation for 30 min. at 37 °C, but not at 45 °C. Anti-248 activity was lost from UBAPF2/pRle306c SF stored for 7 days at -70 °C, -20 °C, 4 °C, 37 °C or 45 °C. This was in contrast to anti-336 activity which was retained by 306SM and 248 SF even inhibit broth cultures of 336 in growth curves conducted over 7 days (Figure 24, Appendix B). Activity was unaffected by filtering the SF through acrodiscs designed not to bind protein (0.22 or 0.45 μm). Bacteriocin activity from 306SM was found to be unaffected by filtration, and the practise of filtering the SF was adopted for all of the SF tests.

Chloroform treated SF from 248 was also inhibitory to 336 in growth curves lasting over 5 days (Figure 25, Appendix B). SF from 306SM treated with chloroform did not have activity against 162Y10 and 248, although activity may have been lost due to extended storage of the SF over several weeks previous to these trials.

Phosphate binds divalent cations such as Ca^{2+} and therefore may have an inhibitory effect on some enzymes. Other buffer components, such as Tris, can also inhibit enzyme activity. In addition, bacteriocin activity from 248 has been shown to be calcium dependent (Oresnik *et al.* 1999). To test the effect of various buffers (pH 7) and salt concentrations on bacteriocin activity, SF samples were incubated with the test solutions at 4 °C overnight before testing. Samples were re-checked for bacteriocin activity after 1 week. The buffers tested affected bacteriocin activity from the test strains differently, with HEPES and PBS causing a lessening of bacteriocin activity from

Table 9. The Effect of Temperature on Bacteriocin Activity of Supernatant Fluid.

Indicator strains	306SM				UBAPF2/pRle306b				UBAPF2/pRle306c			
	RT	37	45	65	RT	37	45	65	RT	37	45	65
248	+	W	-	-	-	-	-	-	++	+	-	-
336	+	+	+	-	W	W	W	-	-	-	-	-

+ inhibition, W weak inhibition, ++ strong inhibition, - no inhibition

Temperature given in degrees Celcius

UBAPF2/pRle306b. Increasing $CaCl_2$ concentrations up to 50 mM did not adversely affect bacteriocin activity in SF from 306SM or UBAPF2/pRle306b, although activity was reduced at a $CaCl_2$ concentration of 250 mM (Table 10). Bacteriocin activity against 248 was strongest at 5 mM $CaCl_2$ as in the test on different media, described above. In contrast, no bacteriocin activity was seen in SF from UBAPF2/pRle306c treated with $CaCl_2$ in concentrations of 50 mM or higher.

The type of medium used in indicator plates has been shown to affect bacteriocin activity, in that strains inhibited on minimal medium are not always inhibited on TY (Hirsch 1979). The bacteriocin activity of 306wt against 248 and 336 on different types of indicator plates was examined (Table 11). Activity against 336 was more pronounced on YEM plates, particularly when supplemented with 5 mM $CaCl_2$. In contrast, no activity was seen against 248 on YEM plates, although activity was seen on both TY and YEM plates supplemented with 5 mM $CaCl_2$. Activity against both indicators was poor in minimal media. Overall, the addition of $CaCl_2$ at a concentration of 5 mM improved bacteriocin activity from 306wt. Soft TY plates supplemented with 5 mM $CaCl_2$ were chosen as the standard medium for making indicator plates.

The results of the above tests indicated that in addition to having different spectra or activity, the bacteriocins encoded on pRle306b and pRle306c had different physical properties which distinguished them as unique from one another.

Table 10. Bacteriocin Activity of Treated Supernatant Fluid

Treatment	Source of Supernatant Fluid		
	306SM	UBAPF2/pRle306b	UBAPF2/pRle306c
Untreated	+	+	+
Buffer (pH 7) :			
Tris-HCl	+	+	+
HEPES	+	W	+
PBS	+	W	+
[Calcium Chloride] a			
0.5 mM	+	+	+
5 mM	+	+	+
50 mM	+	+	-
250 mM	W	W	-

+ inhibits same range of strains as untreated supernatant fluid, -no or little activity, W weakened activity

a Calcium chloride solutions were made up in water and added to SF in a 1:1 ratio. The final salt concentrations are given.

Table 11. The Effect of Medium Used for Indicator Plates on Bacteriocin Activity of 306 wt

Indictator Strains	Medium			
	TY (5mM Ca++)	YEM	YEM (5mM Ca++)	VMM-Glucose
248	+	-	+	S
336	+	+ to ++	++	-

+ inhibition, ++ large halo, S small halo, - no inhibition

Stab tests were used to assay bacteriocin activity.

5.0 Results: Analysis of the Genetic Region Encoding a Novel Bacteriocin from 306

5.1 Two Tn*5* Mutants of 306SM Have Altered Bacteriocin Activity

306 was found to produce three bacteriocins, including a novel bacteriocin encoded on pRle306c. Three different approaches were taken in order to isolate the genetic region coding for this novel bacteriocin from 306.

The first approach was to build a cosmid bank of pRle306c. The plasmid was gel isolated from a preparative Eckhardt gel (a modified Eckhardt with one lane stretching across the entire width of the gel). The isolated plasmid DNA was then partially restricted with *Sau*3A and ligated to cosmid vector pRK7813 (Jones and Gutterson 1987). The ligation products were transformed into *E. coli* DH5α and the resulting clones mated with *A. tumefaciens* UBAPF2 or *R. leguminosarum* 4292 using helper plasmid pRK600. The resulting clones were screened for bacteriocin activity against 248, 336 and VF39. Two putative bacteriocin producing clones were isolated, but restriction analysis of the potential cosmids did not reveal any inserts large enough to see by gel electrophoresis. This is not all that surprising given that the likelihood of ligating smaller fragments together is much greater than the likelihood of ligating larger pieces of DNA, and the genomic DNA was not size fractionated prior to ligation. Small clones are also more likely to be selected for in transformations. Furthermore, in subsequent bacteriocin tests, the clones did not have bacteriocin activity against the indicator strains tested.

The second method was to build a cosmid bank from total genomic DNA from 306SM. Genomic DNA was isolated and partially restricted with *Sau*3A. Conditions were optimised so that DNA fragments were larger than 20 kb, as determined by gel electrophoresis. The genomic DNA was then ligated to pRK7813. *E. coli* DH5α was then transformed with the ligation products to check that ligation was occurring. Although transformants were obtained, indicating that ligation had occurred, when attempts were made to package the DNA using the Promega Packagene λ Packaging

System, no cosmids were obtained. As an alternative to packaging the DNA, 50 transformants were pooled in 5 groups of 10, and mated with 248 and pRK600. 1850 of the resulting clones were screened for the ability to inhibit 248, however, none of the clones showed bacteriocin activity. The transformants screened contained relatively small inserts (10 % had inserts of up to 10 kb, but others had inserts of 3 kb or smaller). Once again, smaller clones were selected for in transformation.

The bacteriocin encoding region in 162Y10 is over 18 kb long (Twelker and Hynes, unpublished: Accession number AF141932). Potentially, the bacteriocin encoding region of interest in 306 might also be very large, and as a result would be extremely difficult to clone out in entirety. (Results from sequence analysis later indicated that this was indeed the case, and that the gene region was well over 11 kb, as described in section 5.3.) 306SM was therefore mutagenised with Tn*5* in order to create a mutation in the bacteriocin encoding region which could then be cloned out into pBSK^{+}. Two thousand isolates of 306SM mutagenised with Tn*5* were tested for their ability to inhibit 248 and VF39 in bacteriocin stab tests. No isolate was found that had altered activity against VF39. One isolate (M1) no longer inhibited 248. Another (M2) produced smaller and less clear halos against 248 compared to the control. The mutation in M1 was later moved into 306DM (described in section 5.6), and 306DM::Tn*5* also showed no activity against 248 (Figure 6). These results were reconfirmed with spot test assays using SF, and it was demonstrated that none of the mutants had lost bacteriocin activity against 162Y10, 336, 3841 or VF39 (Figure 7 and Table 12).

M2 showed noticeably slower growth than M1 on TY plates, while M1 appeared to be phenotypically the same as 306SM, except in bacteriocin production. The plasmid profiles of the mutants (M1 and M2) were checked on Eckhardt gels (Figure 8). Plasmid profiles of the mutants were the same as 306SM or 306DM indicating that no plasmid rearrangements or deletions were likely, and that the bacteriocin- phenotypes observed in

Figure 6. Bacteriocin Activity of 306SM and Tn*5* Mutants Against 248. 306SM produces zones of inhibition on 248 indicator plates, while M2 produces somewhat smaller zones of inhibition on 248 indicator plates. M1 does not inhibit 248. A stab test is shown in which 306SM, M1 and M2 are stabbed into a 248 indicator plate.

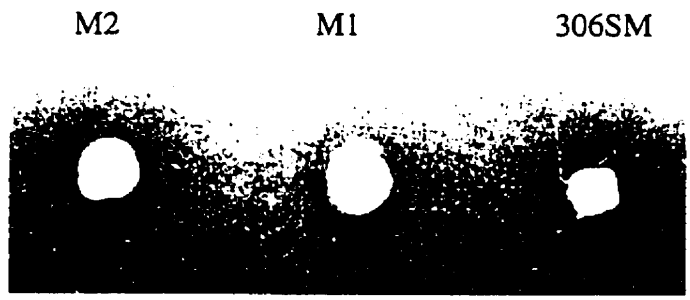
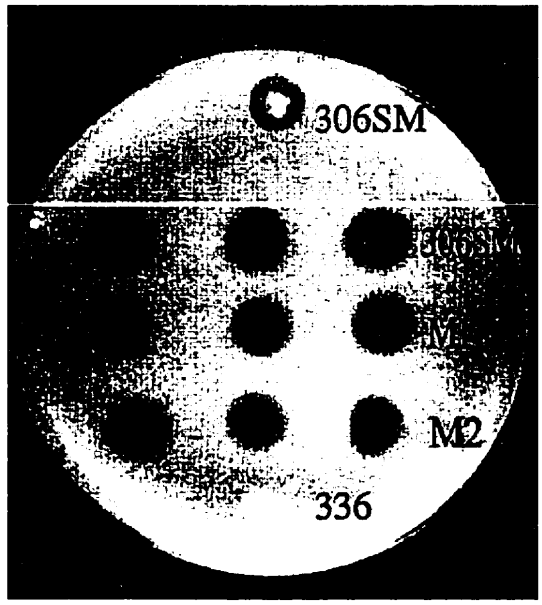
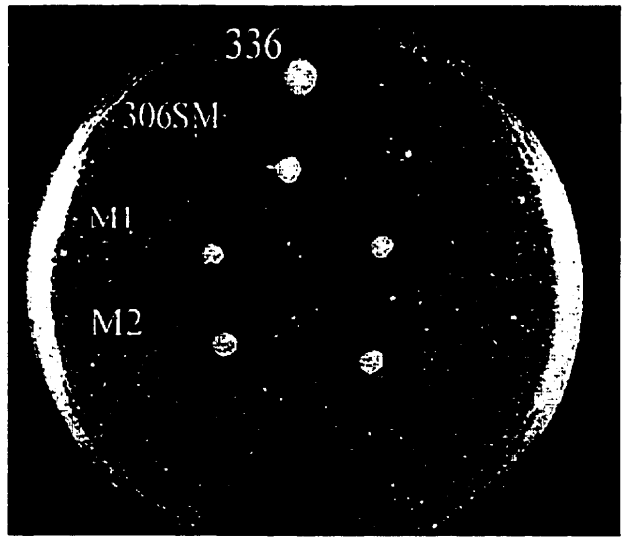

M2
M1
306SM

Figure 7. Bacteriocin Activity of 306SM and Tn*5* Mutants Against 336. 306SM, M1 and M2 produce large, clear zones of inhibition on 336 indicator plates. a. Spot test with SF from 306SM, M1 and M2 on 336 indicator overlay. 306SM is stabbed into the top of the plate as a positive control, and 336 is stabbed into the bottom of the plate as a negative control. b. 306SM, M1, M2 and 336 (negative control) are stabbed into a 336 indicator plate.



a



b

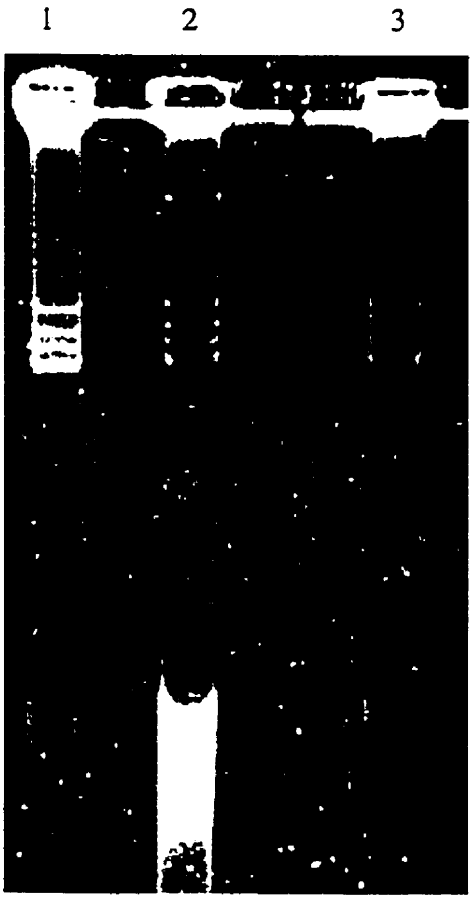
Table 12. Bacteriocin Activity of 306 Tn5 Mutants

Indictator Strains	Strains tested for Bacteriocin Production		
	306	Mutant 1	Mutant 2
248	+	-	W
336	+	+	+
VF39	+	+	+
3841	+	+	+
162Y10	+	+	+

+ inhibition, - no inhibition, W weak inhibition

Stab tests and SF tests were used to detect bacteriocin activity.

Figure 8. Plasmid Profiles of 306SM and Tn*5* Mutants. 306SM and the Tn5 306 mutants have the same plasmid profiles as visualised by a modified Eckhardt gel. 1, 306SM; 2, M1; 3, M2.


1
2
3

the mutants were probably due to the insertion of Tn*5*. This was confirmed by regeneration of the mutation in 306DM (described in section 5.6).

A Southern blot of genomic DNA from M1 (digested with *Eco*RI or *Bam*HI) and M2 (digested with *Eco*RI) was probed with the inner *Bgl*II fragment of Tn*5* (Figure 9). The probe hybridised to different bands in the M1 and M2 *Eco*RI digests, indicating that Tn*5* had inserted in different *Eco*RI fragments in each mutant. The probe hybridised to one band in the *Eco*RI digest lanes and two bands in the *Bam*HI digest lane, indicating that Tn*5* had inserted only once in each mutant (Tn*5* contains one *Bam*HI site and no *Eco*RI sites).

Several attempts were made to localise the Tn*5* insertion to pRle306c by probing Southern blots of Eckhardt gels of the mutants with the inner *Bgl*II fragment of Tn*5*. In each case, specific binding of the probe was not observed. Eckhardt gels contain considerably less DNA than gels containing restriction digests of plasmids prepared by alkaline lysis, and low amounts of DNA on the Southern blots of Eckhardt gels may have accounted for the lack of hybridisation of the Tn*5* probe. An alternative approach might be to run a preparatory Eckhardt gel on UBAPF2/pRle306c and gel isolate pRle306c. The concentrated plasmid sample could be run on an agarose gel, a Southern blot made of the gel, and the blot probed with the inner *Bgl*II fragment of Tn*5*.

5.2 Cloning of DNA Flanking the Tn*5* Inserts

Total genomic DNA was prepared from M1 and M2 and digested with *Eco*RI. The DNA was ligated to pBSK$^+$ and transformed into *E. coli* DH5a. Selection for inserts containing Tn*5* was made by plating transformants on LB-Kan. Southern blots of the putative constructs containing the *Eco*RI inserts were probed with the inner *Bgl*II fragment of Tn*5* (Figures 10 and 11). The probe hybridised to bands corresponding to each *Eco*RI insert in pAVM1 and pAVM2, as well as various restriction fragments from

Figure 9. Southern Blot Demonstrating Insertion of Tn*5* in Different Restriction Fragments in M1 and M2. Total genomic DNA from 306SM, M1 and M2 was restricted and probed with the inner *Bgl*II fragment from Tn*5*. The *Eco*RI fragments from M1 and M2 are different sizes. The probe bound to only one *Eco*RI fragment from M1 or M2 and two *Bam*HI fragments from M1, indicating that Tn*5* inserted only once in each mutant. 1, DNA from M1 digested with *Eco*RI; 2, DNA from M1 and M2 digested with *Eco*RI; 3, DNA from M2 digested with *Eco*RI; 4, DNA from 306SM digested with *Bam*HI (negative control); 5, DNA from M1 digested with *Bam*HI.


1 2 3 4 5

Figure 10. Region of Tn*5* Insertion is Contained by pAVM1. A Southern blot of pAVM1 clones was probed with the inner *Bgl*II fragment from Tn*5*. Uncut and *Eco*RI restricted plasmid DNA from each clone were analysed. The probe bound to DNA from the clones, indicating that the *Eco*RI inserts in the clone contained Tn*5*. 1, pAVM1-1 uncut; 2, pAVM1-1 cut; 3, pAVM1-2 uncut; 4, pAVM1-2 cut; 5, *Hind*III λ ladder (negative control); 6, pAVM1-3 uncut; 7, pAVM1-3 cut; 8, pAVM1-4 uncut; 9, pAVM1-4 cut; 10, pAVM1-5 uncut; 11, pAVM1-5 cut.

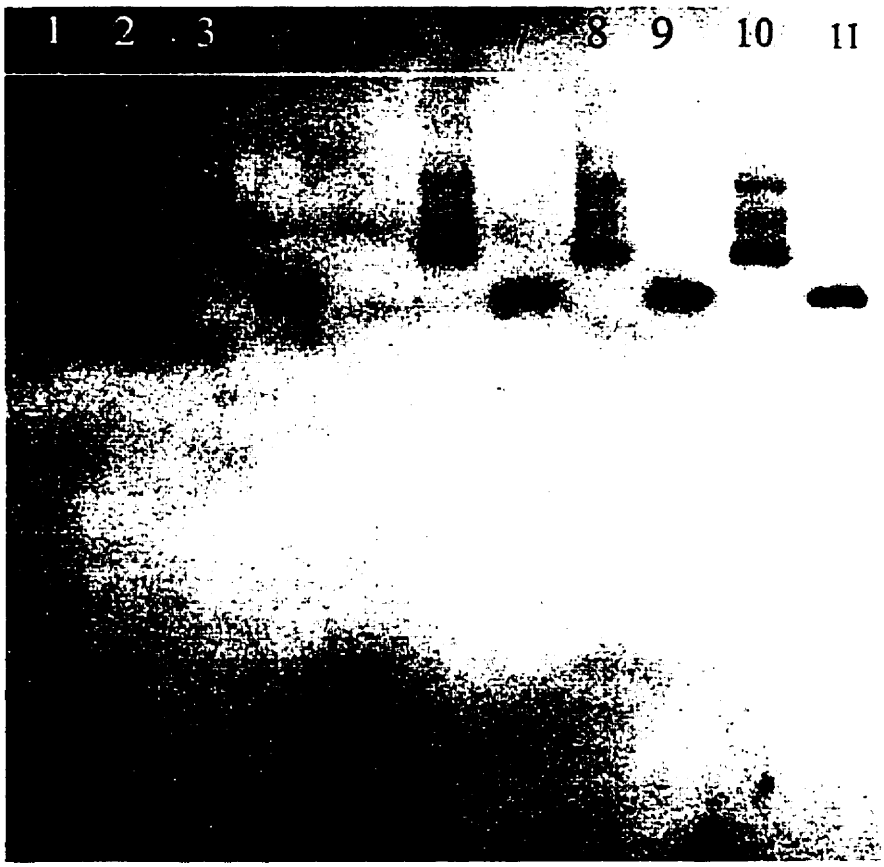

1
2
3
8
9
10
11

Figure 11. Region of Tn*5* Insertion is Contained by pAVM2. A Southern blot of pAVM2 was probed with the inner *Bgl*II fragment from Tn*5* . The probe bound to DNA from the clone, indicating that the *Eco*RI insert contained Tn*5*. One band is seen from DNA digested with *Eco*RI and *Bam*HI, indicating that Tn*5* was located in the middle of the insert. 1, pAVM2 digested with *Eco*RI; 2, pAVM2 digested with *Eco*RI and *Bam*HI; 3, pAVM2 digested with *Bam*HI.

other digests (data not shown), indicating that each insert did indeed contain Tn*5*. Based on the mobility of restriction fragments of the inserts in agarose gels compared to *Hind*III digested λ, the sizes of the *Eco*RI fragments were estimated to be 19.5 kb (pAVM1) and 16.5 kb (pAVM2).

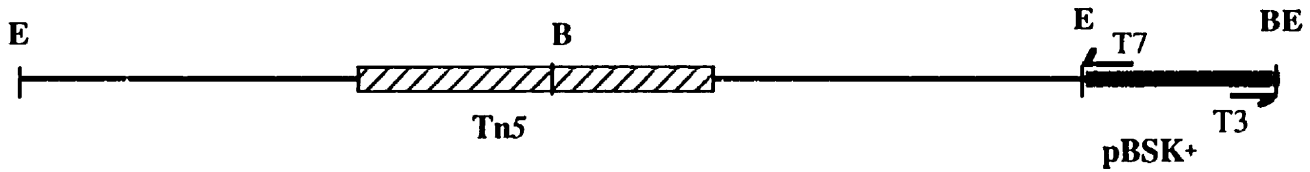
The Tn*5*-containing *Kpn*I fragment from M1 was also cloned into pBSK$^+$ in order to clone out a larger region of DNA. A Southern blot of pAVM3 *Eco*RI and *Kpn*I digests was probed with the inner *Bgl*II fragment of Tn*5*, which hybridised to the insert (Figure 12). The banding pattern of the pAVM3 digests showed that the *Eco*RI insert from pAVM1 was contained by pAVM3 (Figure 12). The size of the *Kpn*I insert contained in pAVM3 was estimated to be 26 kb based on restriction analysis.

5.3 The Regions of Tn*5* Insertion in M1 and M2 Show Homologies to RTX Toxins and Associated Transporters

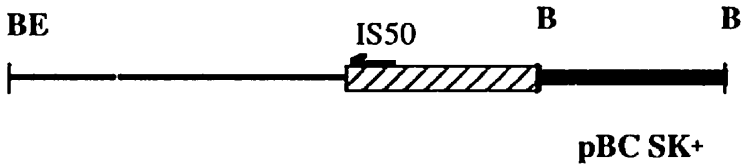
pAVM1 and pAVM2 were sequenced from the T3 and T7 primers of pBSK$^+$ (Figures 13 and 14). DNA sequences were analysed by BLASTX searches for protein homologies to the predicted protein sequences. Search results for sequence from pAVM2 sequenced from the T7 primer showed some homology to chlorohydrolases and hydrolases over short stretches of sequence (300 to 400 bp). The best homology (59% positivity, or similar amino acids and 38% identity, or identical amino acids) was to a *N*-ethylammeline chlorohydrolase from the archeon *Archeoglobus fulgidus* (accession number AE001035) (Klenk *et al.* 1997). Search results for sequence from the other end of the insert showed some homology (55% positivity and 39% identity) over a stretch of 167bp to RTX toxin determinants (Apx IV var 1, 2 and 3) from *A. pleuropneumoniae* (accession numbers AX002405, AX002407 and AF030511 respectively) and FrpA/C from *Neisseria meningitidis* (accession number AAF41768) (Schaller *et al.* 1999; Tettelin *et al.* 2000). This region also showed limited homology (61% positivity and 37% identity

Figure 12. Region of Tn*5* Insertion is Contained by pAVM3. A Southern blot of pAVM3 was probed with the inner *Bgl*II fragment from Tn*5*. The probe bound to DNA from pAVM3, indicating that the *Kpn*I insert contained Tn*5*. The size of the *Eco*RI fragment from pAVM3 was the same as the size of the *Eco*RI insert from pAVM1, indicating that the region of the *Eco*RI insert from pAVM1 was contained by pAVM3. 1, *Hind*III λ marker (negative control); 2, pAVM1 uncut; 3, pAVM3 uncut; 4, pAVM3 digested with *Kpn*I; 5, pAVM3 digested with *Eco*RI; 6, pAVRKM1 (contains *Eco*RI fragment from pAVM1) digested with *Eco*RI.

Figure 13. Restriction Maps of pAVM2 and Subclones. Size estimates are based on banding patterns compared to *Hind*III λ marker. Primers used for sequencing (IS50, T7 and T3) are indicated next to the sequenced clones. Restriction sites are designated as follows: E, *Eco*RI; B, *Bam*HI.



a. pAVM2

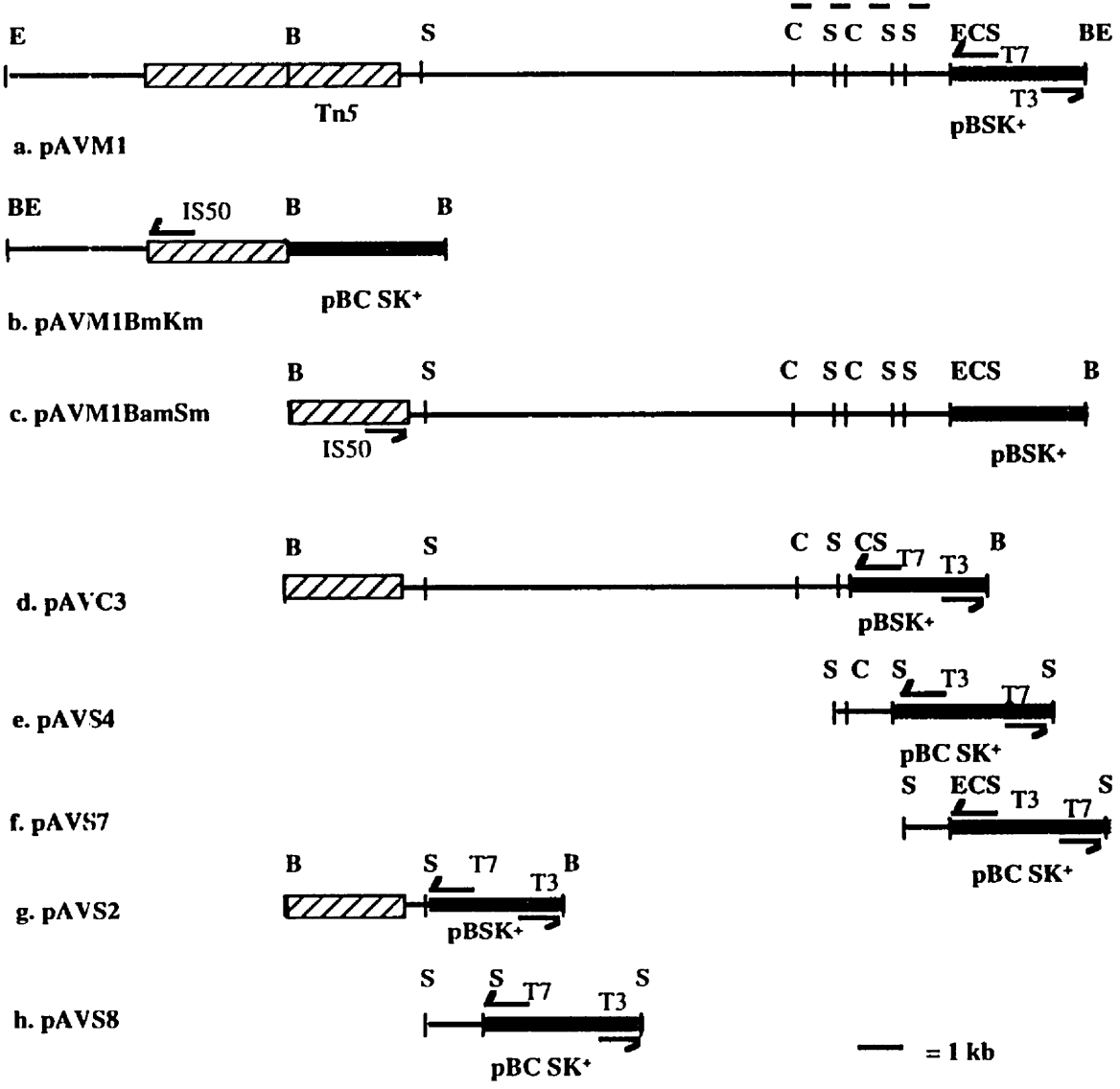


b. pAVM2BamKm

c. pAVM2BamSm

= 1 kb

Figure 14. Restriction Maps of pAVM1 and Subclones. Size estimates are based on sequence data and banding patterns compared to *Hind*III λ marker. The dashed line indicates the region of contiguous sequence containing ORF2. Initial primers used for sequencing (IS50, T7 and T3) are indicated next to the sequenced clones. Restriction sites are designated as follows: E, *Eco*RI; B, *Bam*HI; S, *Sal*I; C, *Cla*I. At least one additional *Sal*I site is present in pAVM1 and pAVM1BamSm, but the exact location of this site is unknown.


E
B
S
C S C S S
ECS
BE
T7
T3
Tn5
pBSK+
a. pAVM1
BE
IS50
B
B
pBC SK+
b. pAVM1BmKm
B
S
C S C S S
ECS
B
c. pAVM1BamSm
IS50
pBSK+
B
S
C S CS
T7
T3
B
d. pAVC3
pBSK+
S C S
T3
S
e. pAVS4
T7
pBC SK+
S
ECS
T3
T7
S
f. pAVS7
pBC SK+
B
S
T7
B
T3
g. pAVS2
pBSK+
S
S
T7
S
T3
h. pAVS8
pBC SK+
= 1 kb

over 173bp) to RzcA, the rhizobiocin produced by 162Y10 (accession number AF141932) (Twelker *et al.* unpublished). No significant homologies were obtained from search results for sequence from subclones of pAVM2 when sequenced from the IS50 primers of Tn*5* (Figure 13).

BLASTX analysis of sequence data from pAVM1 when read from the T3 primer of pBSK$^+$ uncovered no significant homologies. Similarly, BLASTX analysis of sequence data derived from sequencing off of the T3 primer on the *Kpn*I clone, pAVM3, revealed very little. Short stretches of this region (about 150 AA) were homologous to creatine deaminase from different bacteria, as well as the *tfuA* gene from a TFX producing strain of *R. leguminosarum* bv. trifolii (accession number RLU39409) (Breil *et al.* 1996). However, the end of the insert in pAVM1 sequenced from the T7 primer had significant homologies to ABC transporters used by RTX toxins. This region became the focus of further sequence analysis.

Additional sequence data were obtained from the IS50 primer for 2 *Bam*HI subclones of pAVM1 (Figure 14). BLASTX search results for pAVM1BamSm showed low homologies (40% positivity and 25% identity) to regions from a secreted acid phosphatase from *Leishmania mexicana* over approximately 800 bp (accession number Z46970) (Wiese *et al.* 1995). Lower scoring homologies to S-layer proteins from *Caulobacter crescentus* and were also detected (accession number P35828) (Awram and Smit, 1998). No extensive homologies were obtained to sequence from pAVM1BamKm, although some low scoring homologies were detected to *Caulobacter* S-layer proteins and different regions of an RTX protein from *Aeromonas salmonicida* (accession number AF218037). pAVM1BamSm was further subcloned and sequenced (Figure 14). Additional sequence was obtained from pAVM3 and primers designed from the subclones (Figure 15 and Table 13).

A contiguous 4.3 kb region of sequence was obtained. Data from both DNA

Figure 15. Restriction Maps of pAVM3 and Subclones. Size estimates are based on sequence data and banding patterns compared to *Hind*III l marker. The dashed line indicates the region of contiguous sequence containing ORF2 and ORF3. Primers used for sequencing (T7 and T3) are indicated next to the sequenced clones. Restriction sites are designated as follows: E, *Eco*RI; K, *Kpn*I; P, *Pst* I.

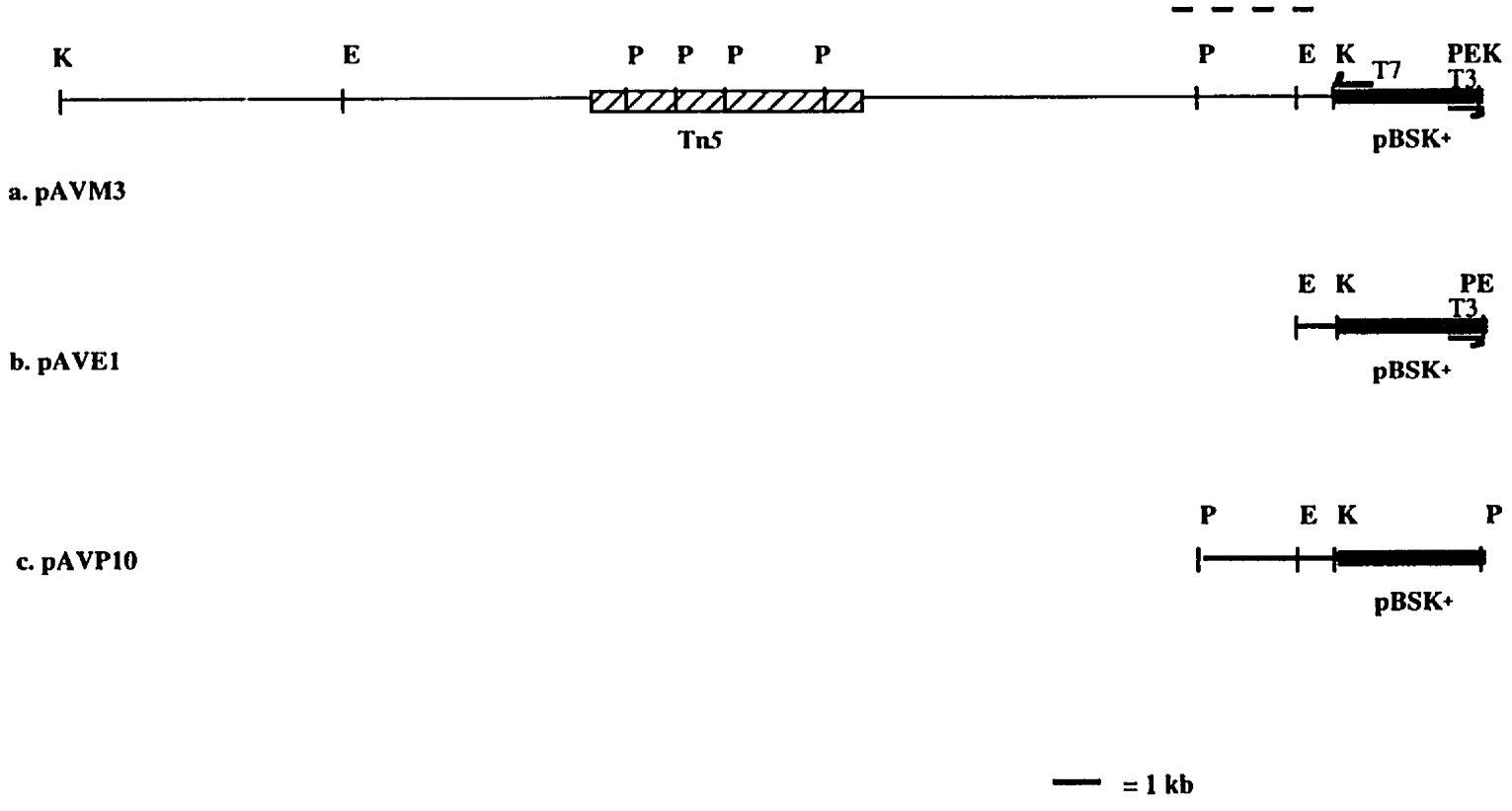

K
E
P P P P
P
E K
T7
PEK
T3
Tn5
pBSK+
a. pAVM3
E K
PE
T3
pBSK+
b. pAVE1
P
E K
P
pBSK+
c. pAVP10
= 1 kb

Table 13. List of Primers

Name	Primer (5' to 3')	Source/Reference
T7	GTAATACGACTCACTATAGGGC	Stratagene Cloning Systems, 1995
T3	AATTAACCCTCACTAAAGGG	Stratagene Cloning Systems, 1995
IS*50* primer	TAGGAGGTCACATGGAAGTCAGAT	Oresnik *et al.*, 1999
C3a	GGTGTTGGTGGAGAGTCG	This study
S4T7	CAGGATTTCCAGCAGGTG	This study
S7T3	TAGCGTCTCGTGTGTTCC	This study
C3b	CCTTTGCTGATGGCTCTA	This study

strands was obtained for 3.5 kb of this sequence stretch, while the rest of the data was based on one strand of DNA (Figure 16). Within the double stranded region a 2205 bp open reading frame (ORF2) was detected. BLASTX analysis of ORF2 revealed extensive homologies (61-62% positivity and 45-48% identity) to several ABC transporters of the HlyB type, including transporters of Apx from *A. pleuropneumoniae* (accession number P26760), a cytolysin from *N. meningitidis* (accession number AE002488), leukotoxin from *Pasteurella haemolytica* (accession number C30169) and haemolysin from *E. coli* (accession number LEECB) (Felmlee *et al.* 1985; Frey *et al.* 1994; Strathdee and Lo 1989; Tettelin *et al.* 2000). (Accession numbers and references given are for those matches having the highest degree of homologies to ORF2). Overall, more than 100 matches to various ABC tranporters were detected. An alignment of the predicted protein for ORF2 with the search result showing the most significant homology is given in Figure 17.

31 bp before the termination codon for ORF2, a partial open reading frame (ORF3) lacking a stop codon was detected. Another potential start site for ORF3 was located overlapping the termination codon from ORF2. Based on a similar putative operon structure for the bacteriocin gene region from *R. leguminosarum* bv. trifolii 162Y10, this second start site was accepted as the start codon for ORF3. Sequence data for ORF3 was double stranded. BLASTX analysis of ORF3 uncovered significant homologies (45-50% positivity and 30-33 % identity) to various HlyD-like transporters of RTX toxins, including secretion proteins NatD from *N. meningitidis* (accession number AE002524), HlyD from *E. coli* (accession number P09986), RtxD from *Vibrio cholera* (accession number AF119150), CyaD from *Bordetella pertussis* (accession number (P11091), LktD from *Actinobacillus actinomycetemcomitans* (accession number P18790), LktD from *P. haemolytica* (M24197) and an Apx secretion protein from *A. pleuropneumoniae* (accession number E43599) (Felmlee *et al.* 1985; Frey *et al.* 1994;

Figure 16. Nucleotide Sequence of Putative Bacteriocin and Transporter Regions from 306. The accession number for the sequence is AF273216. Nucleotides are numbered and the 5' and 3' ends are indicated. Data derived from sequencing only one strand of the DNA are underlined. The remainder of the data shown were derived from both DNA strands. The sequence is contiguous and contains one complete and one partial open reading frame, ORF2 and ORF3 respectively. The putative start site for ORF2 is indicated in bold at base pair 994, and the stop site in bold italics at base pair 3198. ORF2 codes for an HlyB-like ABC transporter. The putative start site for ORF3, shown in bold, overlaps the termination codon for ORF2. ORF3 has homologies to accessory proteins involved in the secretion of RTX toxins. The termination codon for ORF3 is not contained in the region of sequence shown. Preceding ORF2 is a region with homologies to RTX toxins.

5'NGGCNGGCTG GGAGNGTCTC AGTAGAACCT ATATGCTGGG NTCGGCGCAT 50
TGGCAAATGT GTCTCGACAA CGNACGCNCT CGCGTCAGCT TTGATGCGAC 100
GAACACCAAT TCCTGGTGCA ACTCAATATT GTACACAGGT AGCCAACTCG 150
ATTGGCTGCG GCAATATTGG NACNACGGCA CGCTGACACA CCGTTCATAC 200
GACTATGATA CCAGTANCAA CTACTGGCAG TTCGCCTACG ACTACGACGT 250
CAACGGAAAT ACGATCCAAA CCACCAAGTA TTTCGACANC GGTAATTACG 300
ACGTCACGAG AGGCGGTAAG ACCTATCACT ATAATTCGAG CGGACAGTTG 350
ATTGGTCCCG TTCTTCTGGA TCTTGACGGC GACGGCCATG TGGATCTTCG 400
CCCGATCGAT CTGACAAACA TGGCGGCCTC GCCGACGTTC GACTGGGATG 450
GAGACGGTGT TCGCGACACG ACCGCCTGGG CTGGTCCTTC CGATGGCTTC 500
CTTGCCATCG ACCTTGCCTC CGATGGAACG GCCGGTCCCG ATGGCAAGAT 550
CGACCAAGCA AAGGAACTGG CCTTCTCGCT TTGGGCCGAA CAGGACGGGG 600
CGGCAGGGAG TATCTCGGAC CTCGATGGCG TTCGCCTTGT CTTCGATACG 650
AACCACGACA ACGTGCTGGA TTTCAATGAC GAGCGTTGGA ACGAGTTCCG 700
TATTTGGCGT GACGCCAACC AGAATGGTCT CACCGACCAG GGCGAACTTC 750
TGACGATGAC GGACGCCGGC ATCAAGCTCG TCAATTTGAT GCCGACCAGG 800
GATGGCTCGC AGGCCTTTGC TGATGGCTCT ATTATAACGG GAACAAGCTC 850
TTACGAAACT CTGGACGGCT CGAAGCATCT CGTTGCCGAC GCATCACTTA 900
TTTATCGGCC CACCAATGCC ACATAACAAG TTCCCCTGCC TAATGCCGGC 950
GCCTCGCGGC GCCGGAATAC CCTCTCTTCC TTGACGCGAC GCC**ATG**ATGG 1000
ACATCCAGAC AGCATCCGCT GGCGTGGCGG AAGTGGAGAT TAGAGCCGAC 1050
TCTCCACCAA CACCTGAAGC GACAATCGCC GACAGCGGAG TGGCGGCTAT 1100
CTGTGCTGTT GCCGGCTATT TCAGGATCGC ATCGCGTCCG GAGACTCTGA 1150
GCCGTGAGCT TGCGCTGACC GCGCCGGCTG CACGCGACGA CCTGTTGCGG 1200
GCAGCCAAGA TCGTTGGCCT GAAGGCGCGC ACCGTCCGTG CGGAAAAGAT 1250
CTCACGGCTG GCAACGCTTC CGGCGCCCGC CCTTGCTTCG TTGAAGGACG 1300
GCACTTTCGC GGTCTTCGCC GGCCTGGCTG CCGAGGGTCG TTATCGCCTG 1350
ATCAACCCGA TCGATTTCTC GGCCCGCAAT GTCGAGGCCG ACGAGCTTCT 1400
CGCGCTGACC TCGGGCGAGT TCATCCTGGT CCAACGTCGC TTCGCCGGCC 1450
CAGGCGCCTC GCAGCAGAAT TTCGGCTTCC GCTGGTTCCT GCCCGCGATC 1500
TGGCGCTATC GGCGGGCCTT CGGCCATGTG CTGATCGCTT CCCTCGTCAT 1550
CCAGATCTTC GCGCTGGTGA CGCCGCTGTT CTTCCAGGTC GTCGTCGACA 1600
AGGTGCTGGC GCATCGCAGC TATTCGACGC TGATCGTGCT CGTCGTCGGC 1650
CTCGCCGCCG TCGGGCTCTT CGATGTCGTG CTGCAGTATC TCAGAACCTA 1700

TGCGCTGTCG CACACGACCA ATCGCATCGA TGTCGAGCTT GGCCGCCGCC 1750
TTTCCGCCAT CTGTTGAACC TGCCGCTCAG CTATTTCGAA ACCCGCGCCA 1800
CCGGCCAGAC CGTCGCAAGG ATTCGCGAGC TCGAAACCAT CCGCAATTTC 1850
CTCACCGGTC AGGGCCTGTT TTCCGGTCTC GACCTGATCT TCACCATCAT 1900
CTTCATCTTC GTGCTGTTTT CCTATTCGTC GAAGCTCGCC TGGATCGTCG 1950
TCGCTTCGAT CCCCTTTTAC ATGGCAATCG GATTTCTGAT CCGCCCCTTC 2000
CTCAAGGAGC GGATCGACGA GAAGTTCGAA CGCGGCGCCT TCAGCCAGCA 2050
GTTGCTCGTC GAGACCGTCG TCGGCATCCA GACGTTGAAG GCATCGGCGG 2100
TCGAGCCGGT GGTCTCCTCA CAATGGGAAG AGCGGCTGGC CGCCTATGTG 2150
CGCTCGTCGT TTCTCGCGAC CATGCTGGCG GCGAAGGGGC AGAACGCCAT 2200
CCAGTACGTC AACAAGATCA CTTCGGCTGC CCTGCTGCTT TTTGGCGCAC 2250
AGGCGGTGAT CGATGGTGAA CTGAGTGTCG GGGCACTGGT CGCCTTCAAT 2300
ATGATCGCCG GACAGGTCTC CCAGCCGATC CTGCGCCTGT CGCAGCTCTG 2350
GCAGGATTTC CAGCAGGTGC AGGTCTCCAT CGCCAGGCTC TCGGATATTC 2400
TGAATGCGCC GCAGGAACCG CGGCCGAGCG TTGCCGTCAG CCTGCCGCCG 2450
CCGAAGGGGG CGATCGCCTT CAAGTCGGTG AACTTCCGCT ATTCGCCCGA 2500
TGGCCAGGAC GTGCTCAAGG ACATCAATTT TTCGATCCGG CCAGGTGAAG 2550
TGATCGGCAT CGTCGGTCCC TCCGGCTCCG GCAAGTCGAC GCTGACCAAG 2600
CTGGTGCAGC GCTTCTATAT CCCGAACAAC GGCCAGGTCT TCGTCGACGG 2650
CCAGGATATC GCCCAGGTCG ATCCGGCTTG GCTGCGATCC AATATCGGCG 2700
TCGTGCTTCA GGAAAACATG CTGCTGTTCA ACCGGACGAT CCACGACAAT 2750
ATCTGCATGG TCAATCCGGC CATGTCGCGG GCCGCCGCCA TCCAGATGGC 2800
GCGATTGTCC GGTGCCGACC AGTTCATCGC CAAACTGCCG CGCGGCTACG 2850
ACACGCTGAT CGAGGAACGG GGCGCCAACC TCTCCGGCGG CCAAAGGCAG 2900
CGTCTGGCCA TCGCGCGAGC GCTCGCGACC AATCCGCCAA TCCTCATCCT 2950
CGACGAAGCG ACGAGCGCAC TCGATTATGA GAGCGAACGC ATCATCCTCG 3000
GCAATATGCG CGAGATCGTC CGCGGCCGCA CCGTCATCAT CATCGCCCAT 3050
CGCCTGGCAA CCGTCCGACA CTGCAACCGC ATCATCGGCA TGAAGGACGG 3100
ACGCATCGTC GAAGAGGGAA CACACGAGAC GCTACTTGCC CGCCCGAACG 3150
GCCTCTATGC CCATTTGTGG CAATTGCAGA CGGGCCCAAT CGAGTC***ATGA*** 3200
ACCAGACCGT CCAGCCTCCC CGGCTTCCGC CGAAGCCACA GAAGCGATTG 3250
CAGGTGGACA ATGAGTTCCT GCCGGCCGCA CTCGAGATTC TCGAAACGCC 3300
GGCATCGCCG ATCCGCACGG CGCTCATCTG GTTCATCTGC CTGTTCACAG 3350
CCGGCGCACT GATCTGGAGC TATATCGGCA CGTTCGATAT CGTGGCGACC 3400

GCTCAGGGCA AGATCCAGCC GACCGGGCGC GTCAAGGTGA TCCAGTCGAT 3450
CGAGGTTGGC AAGACGATCG CGGTTCCGGT CTCGAACGGT GCCAAGGTGG 3500
AAACCGGAGA CATTCTTGTC GAGCTTGACC CGACCGAAGC CCGGGTCGAG 3550
GTCAACACGC TGACCACCAG CCTCGCGGCA CTGCGGGCAG AAGTCGCGAG 3600
ACGTGGCGCG GCATTGGCTG AGGTTAACGC CTGGCAGCGC GGCGATCTTT 3650
GGAGCGGAAC GCGAAGGATT GAGATCGTCC CGGAATTCGC TGATTCCATA 3700
CCAGCGGAGA TCCGCACGCG GGAGGAACTG CTTTACACGT CCGATCTCGA 3750
GCAGCTCGCC TCGACCCTCG ACAGTCTCGC GGCCCAGCGC AATCAACAAT 3800
TCGCCGCCGT GAAAAGATAT ACCGAGATGG TCACCGCCCA GCGGGCTCTG 3850
GTGGCAACGC TTGCAGACCG TGTCGCCATG CGCTCCAACC TCGTCGATCT 3900
CAGCGCCGGA TCGAGATCCG GCGTCATCGA TGCCGTCGAG ATCAGGCAGA 4000
AGGAAGAGGC AACGCTTGCC GAGCAGATCG GTCAGCGGGC GGAGGCGGAA 4050
ACCGCGATCG TGACGGCAGC GAGCGAAGGC CTGAAAGCCA TCAAGACCTT 4100
CGTCGCGGAC AACGCTGAGA AGCAGGCGGC AGCATCGCGT GAGATCGACG 4150
AGAAGGAACA GCAGCTGGTC AAGGCGGCCA AACGGCTGGA GTCGATGACG 4200
ATCAAAAGCC CGATCAACGG CATTGTCCAG ACATCCGCCA TCACCACGGT 4250
CGGCCAGGTG GTGACCGCCG GCGCCGAACT GATGCGGATC GTCCCGGATG 4300
ATGCTTCTCT TGAGATCGAG GCCTATCTGC CGAACCGAGA CATCGGCTTC 4350
GTCTCACCGG GACAGCCCGC TGTCATCAAG GTCGAAGCTT TCCCATTCAC 4400
CCGCTATGGC ATCCTCAACG GTACC3' 4426

Figure 17. Sequence Alignment of Predicted Amino Acid Sequence for Putative Bacteriocin Transporter Region from 306 with RTX Toxin Determinant B from *A. pleuropneumoniae*. The accession number for the RTX toxin B determinant is P26760. Positive matches (+) and identities (letters) are indicated. The base pair number of the *R. leguminosarum* bv. viciae (RORF2) nucleotide sequence and the amino acid number of the homologous protein sequence (ApxIB) are indicated (Frey *et al.* 1994). Conserved canonical Walker motifs present in the ATP binding domains of ABC transporters are shown in bold letters (Higgins 1992).

```
RORF2: 88   DSGVAAICAVAGYFRIASRPETLSRELALTAPAARDDL---LRAAKIVGLKARTVRAEKI 258
            D G+ A+  +A Y  IA  PE L  +  L       DL   L AAK + LKA+ V+ + I
ApxIB: 8    DYGLYALTILAQYHNIAVNPEELKHKFDLEGKGL--DLTAWLLAAKSLELKAKQVK-KAI 64

RORF2: 259  SRLATLPAPALASLKDGTFAVFAGLAAEGRYRLINPID-FSARNVEADELLALTSGEFIL 435
             RLA +  PAL   +DG   +   + E +  LI  ++  + R +E  E  +L  G+ IL
ApxIB: 65   DRLAFIALPALVWREDGKHFILTKIDNEAKKYLIFDLETHNPRILEQAEFESLYQGKLIL 124

RORF2: 436  VQRRFAGPGASQQNFGFRWFLPAIWRYRRAFGHVLIASLVIQIFALVTPLFFQVVVDKVL 615
            V  R +  G   + F F WF+PA+ +YR+ F   LI S+ +QIFAL+TPLFFQVV+DKVL
ApxIB: 125  VASRASIVGKLAK-FDFTWFIPAVIKYRKIFIETLIVSIFLQIFALITPLFFQVVMDKVL 183

RORF2: 616  AHRSYSTLIVLVVGLAAVGLFDVVLQYLRTYALSHTTNRIDVELGRRLFRHLLNLPLSYF 795
             HR +STL V+ V LA V LF++VL  LRTY  +H+T+RIDVELG RLFRHLL LP+SYF
ApxIB: 184  VHRGFSTLNVITVALAIVVLFEIVLNGLRTYIFAHSTSRIDVELGARLFRHLLALPISYF 243

RORF2: 796  ETRATGQTVARIRELETIRNFLTGQGLFSGLDLIFTIIFIFVLFSYSSKLAWIVVASIPF 975
            E R  G TVAR+REL+ IRNFLTGQ L S LDL+F+ IF  V++ YS KL  +++ S+PF
ApxIB: 244  ENRRVGDTVARVRELDQIRNFLTGQALTSVLDLMFSFIFFAVMWYYSPKLTLVILGSLPF 303

RORF2: 976  YMAIGFLIRPFLKERIDEKFERGAFSQQLLVETVVGIQTLKASAVEPVVSSQWEERLAAY 1155
            YM     I P L+ R+DEKF RGA +Q  LVE+V  I T+KA AV P +++ W+++LA+Y
ApxIB: 304  YMGWSIFISPILRRRLDEKFARGADNQSFLVESVTAINTIKALAVTPQMTNIWDKQLASY 363

RORF2: 1156 VRSSFLATMLAAKGQNAIQYVNKITSAALLLFGAQAVIDGELSVGALVAFNMIAGQVSQP 1335
            V + F  T LA  GQ  +Q++ K+     L  GA  VI G+LS+G L+AFNM++GQV  P
ApxIB: 364  VSAGFRVTTLATIGQQGVQFIQKVVMVITLWLGAHLVISGDLSIGQLIAFNMLSGQVIAP 423

RORF2: 1336 ILRLSQLWQDFQQVQVSIARLSDILNAPQEPRPSVAVSLPPPKGAIAFKSVNFRYSPDGQ 1515
            ++RL+QLWQDFQQV +S+ RL D+LN+P E       ++LP  KG I F+++ FRY PD
ApxIB: 424  VIRLAQLWQDFQQVGISVTRLGDVLNSPTESYQG-KLALPEIKGDITFRNIRFRYKPDAP 482

RORF2: 1516 DVLKDINFSIRPGEVIGIVGPSGSGKSTLTKLVQRFYIPNNGQVFVDGQDIAQVDPAWLR 1695
             +L D+N SI+ GEVIGIVG SGSGKSTLTKL+QRFYIP NGQV +DG D+A  DP WLR
ApxIB: 483  VILNDVNLSIQQGEVIGIVGRSGSGKSTLTKLIQRFYIPENGQVLIDGHDLALADPNWLR 542

RORF2: 1696 SNIGVVLQENMLLFNRTIHDNICMVNPAMSRAAAIQMARLSGADQFIAKLPRGYDTLIEE 1875
              +GVVLQ+N+LL NR+I DNI + +P M      +  A+L+GA +FI++L  GY+T++ E
ApxIB: 543  RQVGVVLQDNVLL-NRSIRDNIALADPGMPMEKIVHAAKLAGAHEFISELREGYNTIVGE 601

RORF2: 1876 RGANLSGGQRQRLAIARALATNPPILILDEATSALDYESERIILGNMREIVRGRTVIIIA 2055
            +GA LSGGQRQR+AIARAL  NP ILI DEATSALDYESE II+ NM +I +GRTVIIIA
ApxIB: 602  QGAGLSGGQRQRIAIARALVNNPKILIFDEATSALDYESEHIIMRNMHQICKGRTVIIIA 661

RORF2: 2056 HRLATVRHCNRIIGMKDGRIVEEGTHETLLARPNGLYAHLWQLQT 2190
            HRL+TV++ +RII M+ G+IVE+G H+ LLA PNGLY +L QLQ+
ApxIB: 662  HRLSTVKNADRIIVMEKGQIVEQGKHKELLADPNGLYHYLHQLQS 706
```

Glaser *et al.* 1988; Guthmiller *et al.* 1990; Highlander *et al.* 1989; Tettelin *et al.* 2000). An alignment of the predicted protein from ORF3 with the search result showing the most significant homology is given in Figure 18. These homologies started 37 bp into ORF3 and lasted until the end of the cloned region of DNA. This evidence and the absence of a stop codon indicated that neither the *Eco*RI insert from pAVM1 nor the *Kpn*I insert from pAVM3 contained the complete gene encoded by ORF3. ORF3 also showed less significant homologies to *prsE* from *R. leguminsarum* bv. trifolii and *S. meliloti* (accession numbers Y12758 and U89163 respectively) (Finnie *et al.* 1997; York and Walker 1997).

BLASTX analysis of a 450 bp region preceding ORF2 revealed significant homologies (45-51% positivity and 29-30 % identity) to the same RTX toxins detected from BLASTX analysis of pAVM2, and the bacteriocin encoding region from *R. leguminosarum* bv. trifolii 162Y10. Sequence data for this region was primarily single stranded. The alignment showing the best fit is given in Figure 19. The homologous RTX toxins included ApxIV var 1, 2 and 3 from *A. pleuropneumoniae* and FrpA/C from *N. meningitidis*. Homologous regions were repeated within RTX toxin proteins and were sometimes overlapping. However, the RTX motif was not detected in the query. The homology with RzcA from 162Y10 (given in Figure 20) continued over a longer region of DNA from the query than did the homologies to RTX toxins (518 bp compared to 461 bp).

5.4 The *Eco*RI Insert Contained by pAVM1 is not Sufficient to Confer Bacteriocin Resistance

The *Eco*RI insert from pAVM1 was cloned into pRK7813 and subsequently mobilised into 248. Clones were selected on VMM glucose plates with Nm or Nm and Tet. Three clones were selected and checked for the presence of the plasmid

Figure 18. Sequence Alignment of Predicted Amino Acid Sequence for Putative Bacteriocin Transporter Region from 306 with Putative Secretion Protein from *N. meningitidis*. The accession number for the *N. meningitidis* protein is AE002524. Positive matches (+) and identities (letters) are indicated. The base pair number of the *R. leguminosarum* bv. viciae (RORF3) nucleotide sequence and the amino acid number of the homologous protein (NatD) sequence are indicated (Tettelin *et al.* 2000).

```
RORF3: 37   KPQKRLQVDNEFLPAALEILETPASPIRTALIWFICLFTAGALIWSYIGTFDIVATAQGK 216
            KP KR   +  FLPA LE+ +TP S        FI  F   AL+WS+ G  DIVA A GK
 NatD: 28   KPPKRTAEEQAFLPAHLELTDTPVSAAPKWAARFIMAFALLALLWSWFGKIDIVAAASGK 87

RORF3: 217  IQPTGRVKVIQSIEVGKTIAVPVSNGAKVETGDILVELDPTEARVEVNTLTTSLAALRAE 396
               GR K IQ +E     AV V +G  V+ G+ L EL+       +V      +L A +
 NatD: 88   TVSGGRSKTIQPLETAVVKAVHVRDGQHVKQGETLAELEAVGTDSDVVQSEQALQAAQLS 147

RORF3: 397  VARRGAALAEVNAWQRGDLWSGTRRIEIVPEFADSIPAEIRTREELLYTSDLEQLASTLD 576
              R  A LA + +                        ++P      +   L  SD +  ++ +
 NatD: 148  KLRYEAVLAALES--------------------RTVPHIDMAQARSLGLSDADVQSAQV- 186

RORF3: 577  SLAAQRNQQFAAVKRYTEMVTAQRALVATLADRVAMRSNLVDLSAGSRSGVIDAVEIRQ- 753
             LA  + Q +AA  +  ++ +A R   A L    A    LV + A  +    D   +R
 NatD: 187  -LAQHQYQAWAA--QDAQLQSALRGHQAELQSAKAQEQKLVSVGAIEQQKTADYRRLRAD 243

RORF3: 754  ---KEEATLAEQI-------------GQRAEAETAIVTAASEGLKAIKTFVADNAEKQAA 885
               E A L +Q              GQ  + + AI  A    +   +    D  +
 NatD: 244  NFISEHAFLEQQSKSVSNWNDLESTRGQMRQIQAAIAQAEQNRVLNTQNLKRDTLDALRQ 303

RORF3: 886  ASREIDEKEQQLVKAAKRLESMTIKSPINGIVQTSAITTVGQVVTAGAELMRIVPDDASL 1065
            A+ +ID+   Q  KA +R + MTI+SP +G VQ  A  TVG VV A  ++M I PDD  +
 NatD: 304  ANEQIDQYRGQTDKAKQRQQLMTIQSPADGTVQELATYTVGGVVQAAQKMMVIAPDDDKM 363

RORF3: 1066 EIEAYLPNRDIGFVSPGQPAVIKVEAFPFTRYGILNG 1176
            ++E  + N+DIGFV  GQ AV+K+E+FP+TRYG L G
 NatD: 364  DVEVLVLNKDIGFVEQGQDAVVKIESFPYTRYGYLTG 400
```

Figure 19. Sequence Alignment of Predicted Amino Acid Sequence for Putative Bacteriocin Region from 306 with RTX Toxin from *A. pleuropneumoniae*. The accession number for the RTX toxin is AX002409. Positive matches (+) and identities (letters) are indicated. The base pair number of the *R. leguminosarum* bv. viciae (RORF1) nucleotide sequence and the amino acid number of the homologous protein sequence (ApxIV) are indicated (Schaller *et al.* 1999).

```
RORF1: 357  PVLLDLDGDGHVDLRPIDLTNMAASPTFDWDGDGVRDTTAWAGPSDGFLAIDLASDGTAG 536
            P+ LDLDGDG   L  + + N      FD +G G+R  T W    DGFL +D   DG
APXIV: 639  PLALDLDGDG---LETVSM-NGRQGALFDHEGKGIRTATGWLAADDGFLVLDRNQDGIIN 694

RORF1: 537  PDGKIDQAKELAFSLWAEQDGAAGSISDLDGVRLVFDTNHDNVLDFNDERWNEFRIWRDA 716
               ++   K           G A +++DLD       TN D  +D ND+ +++ +IWRD
APXIV: 695  DISELFSNKNQLSDGSISAHGFA-TLADLD-------TNQDQRIDQNDKLFSKLQIWRDL 746

RORF1: 717  NQNGLTDQGELLTMTDAGIKLVNLMPTRDGSQAFADGSIITGTSSYETLDGSKHLVADAS 896
            NQNG ++  EL ++    IK ++     + +   A  +I+     YE  DG+   + D +
APXIV: 747  NQNGFSEANELFSLESLNIKSLH-TAYEERNDFLAGNNILAQLGKYEKTDGTFAQMGDLN 805

RORF1: 897  LIYRP 911
              + P
APXIV: 806  FSFNP 810
```

Figure 20. Sequence Alignment of Predicted Amino Acid Sequence for Putative Bacteriocin Region from 306 with RzcA from *R. leguminosarum* bv. trifolii 162Y10. The accession number for RzcA is AF141932 (Twelker *et al.*, unpublished). The base pair number of the 306 nucleotide sequence (RORF1) and the amino acid number of the homologous protein sequence (RzcA) are indicated.

```
RORF1: 321  TYHYNSSGQLIGPVLLDLDGDGHVDLRPIDLTNMAASPT-FDWDGDGVRDTTAWAGPSDG 497
            T HY   GQ   P++LDLDGDG      I+LT +  S +  D D D   +++ + G  DG
 RzcA: 960  TGHYPWGGQ--DPLVLDLDGDG------IELTALDRSKSKLDLDDDLYAESSGFVGKDDG 1011

RORF1: 498  FLAIDLASDGT-AGPDGKIDQAKELAFSLWAEQDGAAGSISDLDGVRLVFDTNHDNVLDF 674
             L  DL  DG   G D     A     +  A  DG      D +  R + D N D  +
 RzcA: 1012 VLVRDLNGDGKITGSDEMFGNATTSGLAALALLDGNHDGKVDAND-RGLADFNGDGAVTA 1070

RORF1: 675  NDERWNEFRIWRDANQNGLTDQGELLTMTDAGIKLVNLMPTRDGSQAFADGSIITGTSSY 854
             D  ++   +W+DAN+N  TD GEL  + + GI  +N+ P    +    +G+ I  T+ Y
 RzcA: 1071 ADS-FSSLLVWQDANENHRTDAGELKGVVERGIASINVTPDTATTTQTVNGNTIASTAGY 1129

RORF1: 855  ETLDGSKHLVADASLIYRPTNAT 923
               DG+   +AD  L     N T
 RzcA: 1130 TLADGTVRTIADVVLKLDNQNTT 1152
```

(pAVRKM1). The insert and vector were clearly visible in agarose gels of *Eco*RI digests of plasmid DNA extracted directly from the clones. The plasmid profiles of the clones on a modified Eckhardt gel looked normal, indicating that no plasmid rearrangements had taken place.

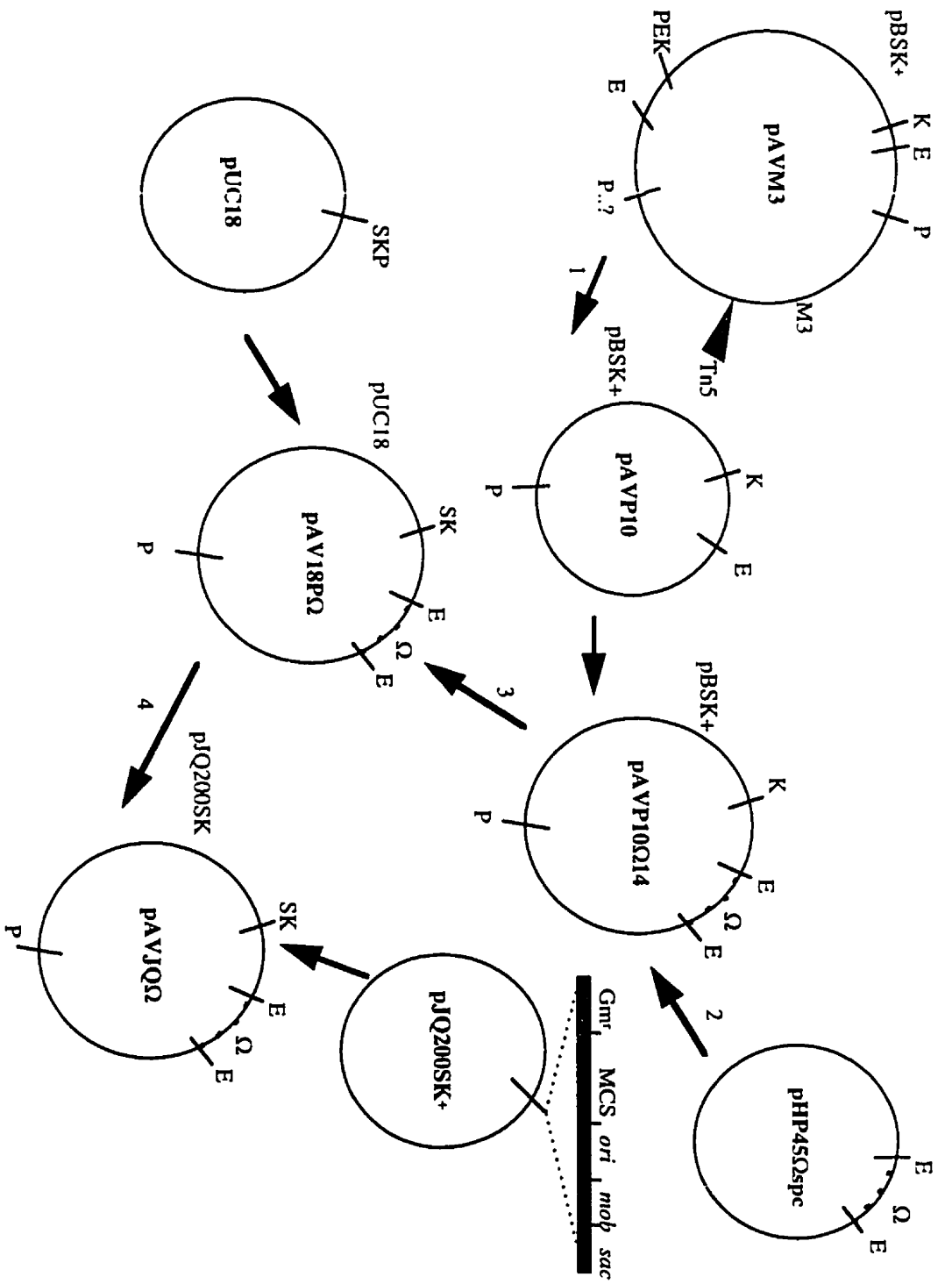
It was observed that the clones grew very slowly in broth culture, reaching saturation only after several days at 30 °C.

Each clone was tested for susceptibility to bacteriocin activity from 306wt, 306SM, and 306SM/RP4 (Tet^r). Tet was added to some of the indicator plates to ensure plasmid maintenance in the indicator strains, and comparisons were made to indicator plates without antibiotics. 306wt and 306SM stabs produced zones of inhibition on the indicator plates without antibiotics, but not on the Tet plates. 306SM/RP4 produced zones of inhibition against all of the clones, in both Tet containing plates and plates without antibiotics. Since clones grown in the presence of Tet would not be expected to lose pAVRKM1, it appears that in 248, this plasmid does not confer immunity to the bacteriocin produced by 306SM having anti-248 activity.

5.5 Introduction of a Mutation into the Putative Bacteriocin Transporter Region

In order to show that the transporter region cloned in pAVM1 was responsible for the secretion of bacteriocin from 306, a mutant was created with an interruption in the *hlyD*-like gene. Gene-replacement was used to create this mutation. First, pAVM3 was digested with *Pst*I and religated (Figures 15 and 21). Potential subclones were always checked by restriction analysis to ensure that the inserts of interest were contained by the clones. The Ω cassette from pHP45Ωspc was then introduced into the unique *Eco*RI site in the *Pst*I subclone. Because the intermediate clone (pAVP10Ω14) did not have restriction sites that were compatible with the suicide vector to be used, the insert containing the Ω cassette was cloned into pUC18 in *Kpn*I and *Pst*I. The insert was then

Figure 21. Construction of Transporter Mutants. 1. pAVM3 was digested with *Pst*I and religated to create pAVP10. 2. The Ωspc cassette from pHP45 Ωspc was introduced into a unique *Eco*RI site in pAVP10. 3. The region flanking the cassette was cloned into the *Kpn*I and *Pst*I sites in pUC18. 4. The insert was then cut out with *Sst*I and *Pst*I, and cloned into these sites in the gene replacement vector, pJQ200SK$^+$ The cassette was introduced into 306DM and 306SM using the gene replacement vector. 306DM was mated with MT616/pRK600 and DH5a/pAVJQM1, and 306DM::Ω clones were selected for on VMM-Spc/sucrose (5%). 306SM was also mated with MT616/pRK600 and DH5a/pAVJQM1, and 306SM::Ω clones were selected for on TY/Spc/Sm/ sucrose (5%). Clones were screened for loss of Gm^r to ensure that double crossovers had occurred, and that the vector was not contained in the 306 clones. Plasmids and multiple cloning sites (MCS) are not drawn to scale. Only the relevant restriction sites are indicated: E, *Eco*RI; K, *Kpn*I; P, *Pst*I, S, *Sst*I. pAVM1 contains additional *Pst*I sites which are not shown, but none are between the *Pst*I site marked and the closest *Kpn*I site. (Prentki and Krisch 1984; Quandt and Hynes 1993).


pBSK+
K E P
PEK
E
P..?
pAVM3
M3
Tn5
1
pBSK+
pAVP10
K
P
E
pUC18
SKP
pUC18
pAV18PΩ
SK
P
E
Ω
E
3
pBSK+
pAVP10Ω14
K
P
E
Ω
E
2
pHP45Ωspc
E
Ω
E
Gmr
MCS
ori
mob
sac
pJQ200SK+
4
pJQ200SK
pAVJQΩ
SK
P
E
Ω
E

cut out with *Sst*I and *Pst*I and cloned into these sites in the gene replacement vector pJQ200SK$^+$. The resulting plasmid, pAV18PΩ, was transformed into *E. coli* DH5α. DH5α/pAV18PΩ was mated with 306DM or 306SM, and helper plasmid pRK600. 306DM mutants were selected on VMM sucrose 5% with Spc. Since it was difficult to obtain pure cultures using only VMM as a method of selection against *E. coli*, 306SM mutants were selected for on TY sucrose 5% with Spc and Sm.

Putative transporter mutants were screened for loss of bacteriocin activity against 248. In stab tests using 248 as an indicator strain the putative mutants gave slight halos, but in spot tests using SF no bacteriocin activity was detected (Table 14). Four 306SM mutants, Ω1, Ω2, Ω4 and Ω8 were isolated as well as one 306DM mutant, Ω12. The putative transporter mutants had normal activity against 162Y10 and 336. These results indicated that the transporter knocked out in the mutants was necessary for the expression of bacteriocin activity against 248.

5.6 A 306 Bacteriocin Minus Mutant Competes Equally Well for Nodulation as the Wild Type

It was of interest to investigate the role bacteriocin production by 306 plays in competition between strains. As a preliminary study, the ability of 306SM to compete with 248 and 162Y10 in broth culture compared to the ability of the mutants, M1 and M2, to compete with 248 and 162Y10, was examined. Both mutants would be expected to compete as well as 306SM against 162Y10, since they both inhibit 162Y10 to the same extent as the parent strain. If bacteriocin production by 306SM were to play a significant role in inter-strain competition, the mutants would be expected to compete less well with 248 than the parent strain, since 248 is not inhibited by M1 and is inhibited only to a limited extent by M2. 306SM was also competed against each of the mutants and against 306wt. Competing cultures were inoculated in 1:1 ratios in TY broth for a final dilution

Table 14. Bacteriocin Activity of Putative Transporter Mutants

Indictator Strains	Strains tested for Bacteriocin Production		
	306	306SM Mutants	306DM Mutant
248	+	-	-
336	+	+	+
162Y10	+	+	+

+ inhibition, - no inhibition

of 1/500, and incubated on a roller for 2 days. Initial cell numbers used were verified by plate counts and these values were taken into account when evaluating the data. The expected frequencies used in calculating χ^2 values were determined by multiplying the number of colonies scored by the inoculation ratio (one strain to both strains in a particular trial). Following competition, aliquots from each test were diluted and plated out to determine a final plate count. Approximately 200 colony forming units (CFU) from each test were patched on TY plates and selective media. Strains were distinguished based on their ability to grow on the selective media (i.e. 306SM is Sm^r, and M1 and M2 are Nm^r and Sm^r). Controls were run to ensure that the strains used had similar growth rates. To this end, final plate counts of the controls (each strain by itself) were compared. All of the strains reached a final population density of 10^9 CFU/mL after 48 hours, indicating that these strains probably had similar growth rates.

In the broth competition experiment, 306SM, M1 and M2 were all highly successful at competing with 248 and 162Y10 in broth culture (Table 15). χ^2 analysis was used to determine if the differences between the competing strains were significant. In this experiment, bacteriocin activity against 248 did not appear to contribute to the competitive ability of 306SM, since the mutants were also very competitive. It was not possible to use χ^2 analysis to compare the ability of 306SM to compete with 248 or 162Y10 to the ability of the mutants to compete with 248 or 162Y10, since no 248 or 162Y10 colonies were recovered in competitions with 306SM. The observed frequencies of the wild type would normally be used to calculate the expected frequencies of the mutant in a different trial, however, this was impossible as 0 is indivisible by numbers other than 0, and 0/200 colonies were obtained for 248 and 162Y10 when either was competed against 306SM. Had a higher number of colonies had been screened, it is possible that some 248 or 162Y10 colonies would have been found, in which case, χ^2 analysis of the differences between trials could have been calculated. Nevertheless, the

Table 15. Broth Competition Assays for 306SM, 306wt and Bacteriocin Mutants

Competition	Inoculation Ratio [a]	Colonies Scored	Colonies with Indicator Strain [b]	% Colonies Scored with Indicator Strain	χ^2 [c]
306SM/306wt	2.5 / 2.21	196	159	81.12	61.8931
306SM/M1	2.5 / 1.81	200	98	49	6.6573
306SM/M2	2.5 / 1.56	197	94	47.72	15.9963
306SM/248	2.5 / 1.05	200	200	100	84
M1/248	1.81 / 1.05	200	193	96.5	94.955
M2/248	1.56 / 1.05	200	194	97	115.287
306SM/162Y10	2.5 / 1.31	200	200	100	104.8
M1/162Y10	1.81 / 1.31	200	200	100	144.7514
M2/162Y10	1.56 / 1.31	199	191	95.98	138.9693

[a] Inoculation ratios were derived from initial plate counts. [b] Indicator strains are underlined in competition column. [c] χ^2 values shown are for the difference between competing cultures in a given trial. For P values of 0.005 or greater, there is no significant difference between 306SM and M1, while the differences between all other trials are significant. The χ^2 value could not be obtained to test the significance of the difference between the wild type and mutants in different trials, because none of the colonies screened in the competition trials using 306SM vs. 248 and 306SM vs. 162Y10 were derived from 248.

data indicated that 306SM and the mutants out-competed 248 and 162Y10 to approximately the same extent in broth culture.

306SM out-competed 306wt to a significant extent, based on χ^2 analysis. 306SM also out-competed M2 to a lesser, although significant extent. In competitions where the M1 was competed against 306SM, M1 and the parent strain competed equally well, and there was no significant difference found between them.

Inter-strain competition in broth culture may not reflect the ability of different strains to compete for nodulation. Plant competition experiments were therefore used to evaluate the role of bacteriocin production by 306SM in this strain's ability to compete for nodulation. Initial plant competition experiments were carried out with Trapper peas. 306SM, M1 and M2 were tested for their ability to compete for nodulation against 248 and against one another. Plants not inoculated with 248 were stunted and chlorotic, indicating that 306SM and the mutants were Fix^- on Trapper peas. In addition, nodules produced by 306SM and the mutants were extremely small, round and white, while nodules produced by 248 were large, elongated and red. Plants inoculated with 248 were lush and healthy, indicating that 248 was Fix^+, as expected. To confirm the Fix^- phenotype observed for 306SM, a number of 306 strains were used to inoculate Trapper peas and vetch. 306SM, M1, M2, 306 wt, 309SM and 309wt were all found to be Fix^- on both Trapper peas and vetch.

To determine the number of nodules occupied by the various strains in the competition experiment, nodules were harvested, surface sterilised, crushed, and the nodule squash spotted on TY and selective media. Strains occupying the nodules were identified by the ability to grow on selective media. When competed against 248, the proportion of nodules occupied by 306SM or the mutants was extremely low (less than 14 %). In light of the Fix^- phenotype of the 306 strains, it seemed prudent to repeat the experiment using Fix^+ 306 strains in order to eliminate differences due to nitrogen fixation

ability. In addition, the uninoculated control contained over 40 nodules, indicating that a considerable amount of cross-contamination may have occurred between the different trials.

In order to repeat the plant experiment with Fix^+ strains, the Tn*5* mutation from M1 was introduced into the Fix^+ strain, 306DM (Figure 22). The *Eco*RI insert from pAVM1 was cloned into the *Eco*RI site in pRK7813, resulting in a Tet^r clone (pAVRKM1). At each step, transformants were checked by restriction analysis to ensure that the insert of interest was present in the subclones. In order to clone the *Eco*RI fragment into the suicide vector, pJQ200SK$^+$, flanking *Xba*I sites were required. Therefore, the insert from pAVRKM1 was cloned into the *Eco*RI site in pUC1819 to create pAV1819M1 (Schweizer 1993). Tet sensitive and Amp resistant white clones were selected. Using the flanking *Xba*I sites, the insert was cloned out of pAV1819M1 and into pJQ200SK$^+$ to create pAVJQM1. DH5α/pAVJQM1 was mated with 306DM and helper strain pRK600. 306DM mutants were selected on VMM sucrose 5% containing Nm, and cross-checked for the loss of Gm resistance. Double cross-overs were thus apparently obtained in which the Tn*5* mutation from M1 had been introduced into 306DM.

Genomic DNA was isolated from the potential mutants and digested with restriction enzymes. A Southern blot of the restriction digests of M1 and putative 306DM mutants was probed with the inner *Bgl*II fragment from Tn*5* (Figure 23). The probe bound to the same *Cla*I and *Eco*RI fragments in M1 and the putative mutants, confirming that the Tn*5* mutation had been reintroduced into the mutants (306DM::Tn*5* isolates 6, 7 and 9). The plasmid profile of 306DM::Tn*5* looked like the parent strain on an Eckhardt gel, indicating that no plasmid rearrangements had taken place. Also, 306DM::Tn*5* did not produce halos against 248, but otherwise inhibited the same strains as 306DM or 306SM.

Figure 22. Construction of 306DM::Tn*5*. 1. The *Eco*RI insert from pAVM1 was cloned into the *Eco*RI site in pRK7813 to create a Tet^r clone. 2. The *Eco*RI insert was then cloned into the *Eco*RI site in pAV1819M1, and Amp^r clones were selected. 3. The insert was cut out from flanking *Xba*I sites in the vector, and cloned into the *Xba*I site in the gene replacement vector, pJQ200SK$^+$. The Tn*5* mutation in M1 was introduced into 306DM using gene replacement vector pJQ200SK$^+$. 306DM was mated with MT616/pRK600 and DH5a/pAVJQM1, and 306DM::Tn*5* clones were selected for on VMM-Nm/sucrose (5%). Clones were screened for the loss of Gm^r to ensure that double cross-overs had occurred, and that the vector was not contained by the 306DM::Tn*5* clones. Plasmids and multiple cloning sites (MCS) are not drawn to scale. Only the relevant restriction sites are indicated: E, *Eco*RI; X, *Xba*I (Quandt and Hynes 1993).

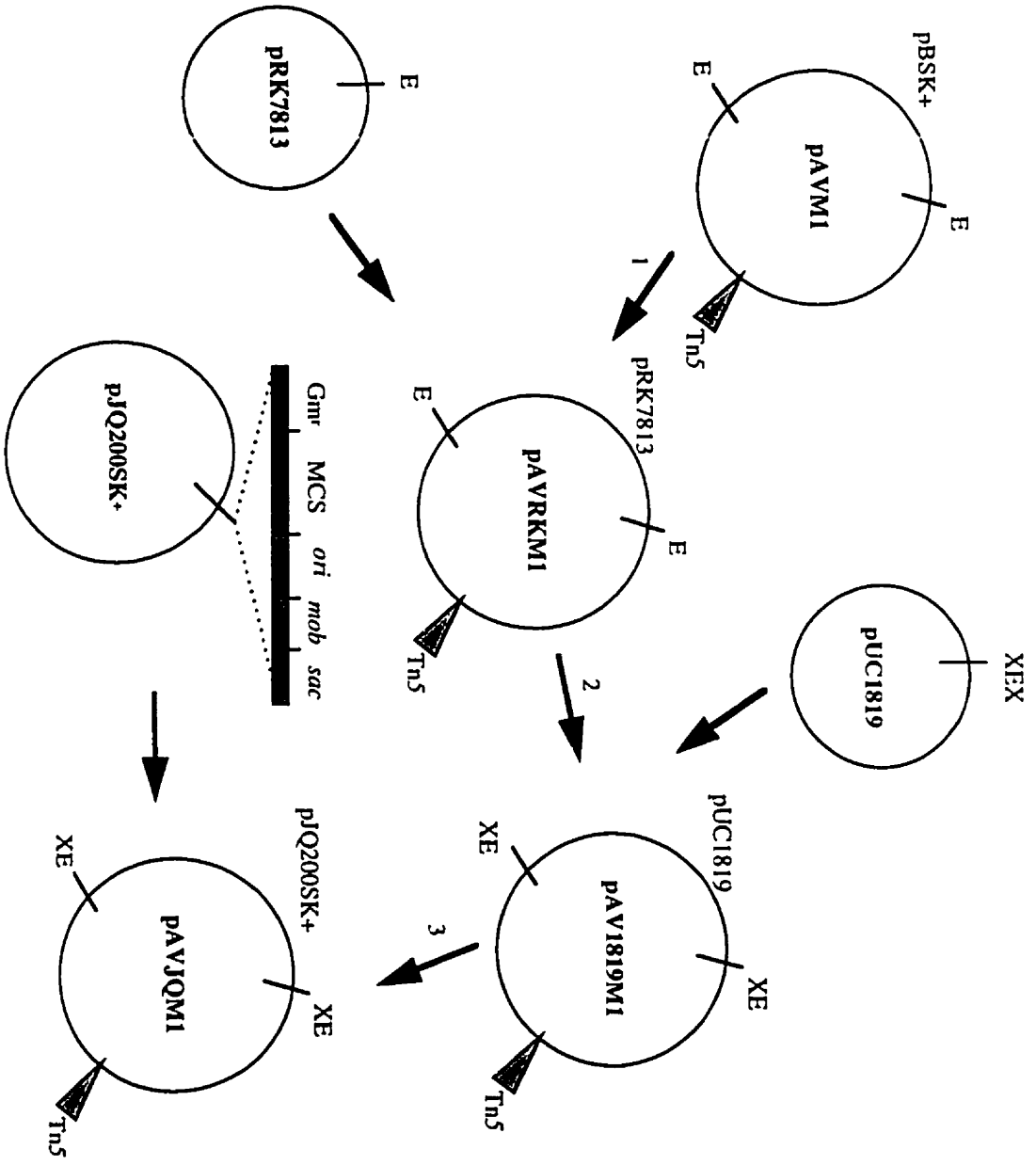
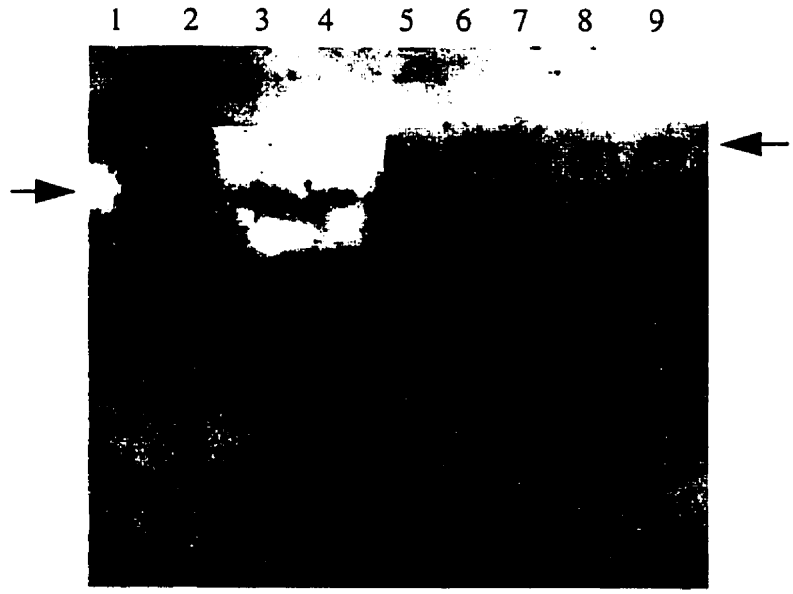

pBSK+
E
pAVM1
E
Tn5
1
pRK7813
E
pAVRKM1
E
Tn5
2
pUC1819
XEX
pUC1819
XE
pAV1819M1
XE
Tn5
3
pJQ200SK+
XE
pAVJQM1
XE
Tn5
pRK7813
E
E
pJQ200SK+
Gmr
MCS
ori
mob
sac

Figure 23. Tn*5* Mutation from M1 is Also Present in 306DM::Tn*5*. A Southern blot of genomic DNA from M1 and 306DM::Tn*5* clones was probed with a Tn*5* probe. The image has been digitally enhanced to make the hybridising bands stand out. 1, M1 cut with *Cla*I; 2, 306DM::Tn*5*-6 cut with *Cla*I; 3, 306DM::Tn*5*-7 cut with *Cla*I; 4, 306DM::Tn*5*-9 cut with *Cla*I; 5, 306SM cut with *Eco*RI; 6, M1 cut with *Eco*RI; 7, 306DM::Tn*5*-6 cut with *Eco*RI; 8, 306DM::Tn*5*-7 cut with *Eco*RI; 9, 306DM::Tn*5*-9 cut with *Eco*RI.


1 2 3 4 5 6 7 8 9

In a new plant experiment, 306DM and 306DM::Tn*5* were competed against 248 or the bv. viciae strain 3841 for the nodulation of Trapper pea plants. Since the mutation in 306DM::Tn*5* did not affect bacteriocin activity against 3841, 306DM and 306DM::Tn*5* were expected to compete equally well for nodulation against 3841. The 3841 competition trials were therefore used as controls. The role of bacteriocin production against 248 was assessed by competing 306DM or 306DM::Tn*5* against 248. 306DM and the mutant were also competed against each other as a control. Plants were inoculated with 1:1 ratios of the competing cultures, and following the duration of the trial, nodules were removed from the plant, surface sterilised and crushed. No nodules were detected on the uninoculated controls, indicating that cross-contamination in the growth chamber was unlikely. Aliquots from the crushed nodules were then spotted on TY plates and selective media. Strains were identified by their ability to grow on selective media (i.e. 306DM::Tn*5* is Nm^r, 3841 is Sm^r, and 306DM is Kan^r at 10 μg/mL). 248 and 306DM were also distinguished by the ability of 306DM to produce halos on 248 indicator plates. Since 306DM grew poorly on the Kan plates, the results of the bacteriocin screen were accepted over the results from the selective media in trials in which there was a discrepancy between the two tests. Twelve of the recovered strains were screened for plasmid rearrangements on an Eckhardt gel, and no alterations in the plasmid profiles were observed.

306DM and 306DM::Tn*5* were much less competitive than 3841, although the mutant was slightly but significantly more competitive than the wild type as determined by χ^2 analysis (Table 16). Both 306DM and the mutant were out-competed by 248, but the wild-type occupied a higher percentage of the nodules screened (27.07 %) compared to the mutant (21.58 %). However, this difference was found to be insignificant based on χ^2 analysis and an analysis of variance using Systat. Starting inoculum ratios were

Table 16. Nodulation Competition Assays of 306 and Bacteriocin⁻ Mutant

Competition	Inoculation Ratio[a]	Nodules Scored	Nodules with Indicator Strain[b]	% Nodules with Indicator Strain	χ^2 [c]
306DM / 306DM::Tn5	2.78 / 2.0	60	31	51.67	1.0393
306DM / 248	2.78 / 2.49	181	49	27.07	47.8886
306DM::Tn5 / 248	2.0 / 2.49	190	41	21.58	40.563
306DM / 3841	2.78 / 0.92	121	8	6.61	304.1138
306DM::Tn5 / 3841	2.0 / 0.92	126	23	18.25	147.3681

[a] Inoculation ratios were derived from plate counts. [b] Indicator strains are underlined in competition column.
[c] χ^2 values shown are for the difference between competing cultures in a given trial. For P values of 0.100, there is no significant difference between the nodule occupancy of 306DM vs. 306DM::Tn5. The χ^2 value was also determined for the difference between the wild type and mutant in different trials. There was no significant difference between nodule occupancy of 306DM and 306DM::Tn5 when competed against 248 (χ^2= 1.279) at a P value of 0.100. 306DM::Tn5 occupied significantly more nodules than the wild type when competed against 3841 (χ^2=180.3547) at a P value of 0.005.

verified by plate counts, and taken into account in χ^2 analysis of the results, as in the broth competition experiment. The differences noted between strains in the same trial were found to be significant at a P value of 0.005, except in the competition trial between 306DM and the mutant, in which both strains were found to compete equally well with one another. This difference was found to be insignificant as tested by χ^2 analysis at a P value of 0.100.

Throughout the run of the experiment it was noted that plants inoculated with 3841 or 248 were faster to develop than plants inoculated with only 306DM or 306DM::Tn*5*. Nodules from plants inoculated with 248 and 3841 were generally large, elongated and dark, compared to the small, round, white-green nodules recovered from plants solely inoculated with the 306 strains. This indicated that the nodules produced by 248 and 3841 were considerably older than the nodules produced by 306DM and the mutant. In fact, many nodules recovered from plants inoculated with 248 were beginning to senesce, although these nodules were removed within 4 days of harvesting nodules from the other plants. Clearly, 306DM and the mutant were slower to nodulate the pea plants than 3841 or 248.

6.0: Discussion

6.1 Bacteriocins Produced by *R. leguminosarum* bv. viciae Strain 306

Previously it was determined that strain 306 produces two bacteriocins, one of which is similar to that produced by 248 (Hirsch 1979; Oresnik *et al.* 1999). The results of the current study indicate that 306 produces at least three bacteriocins, and that the bacteriocin having activity against 248 is a novel bacteriocin. The genetic region encoding this novel bacteriocin was further characterised by sequence analysis, and the role of this bacteriocin in competition for nodulation between different strains was examined.

The genetic determinants for two of the bacteriocins detected by Hirsch (1979) are plasmid encoded. The bacteriocin produced by both 248 and 306 has activity against small bacteriocin producers such as 336 (Hirsch 1979; Oresnik *et al.* 1999; This study). This bacteriocin is encoded on self-transmissible plasmids, pRl1JI, in 248, and pRle306b (pRl3JI), in 306 (Hirsch 1979; This study). In this study it was confirmed that the bacteriocin having activity against 248 is encoded on pRle306c. However, the bacteriocins encoded on pRle306b and pRle306c do not account for all of the bacteriocin activity of 306. 306 also has activity against 162Y10, and it has been noted that halo production by 306 on 336 indicator plates is much more pronounced than halo production from UBAPF2/pRle306b. Each 306 plasmid has been mobilised into UBAPF2 and the transconjugants tested for bacteriocin activity, but none of these plasmids has been found to confer activity against 162Y10. It is possible that the genes coding for bacteriocin production against 162Y10 were interrupted by Tn-*mob-sac* in the transposon tagged plasmids. However, given the large size of the plasmids it is unlikely that the transposon would have inserted in the bacteriocin encoding genes at a high frequency. The bacteriocin genes on pRle306b and pRle306c (coding for bacteriocin activity against 336 and 248 respectively) were not interrupted in any of the tagged isolates used in the study, and these isolates represent several independent mutations.

The genetic determinants for the third 306 bacteriocin could be chromosomally encoded as are the genes for TFX and small bacteriocins (Hynes and McGregor 1990; Triplett 1988). However, the third 306 bacteriocin is unlikely to be a small bacteriocin of the classical kind since, as a medium bacteriocin producer and small sensitive strain, 306 would be expected to carry the genes for the repression of small bacteriocins. In addition, 162Y10 is not small sensitive, while it is sensitive to bacteriocin activity from 306. In 248, the genes coding for sensitivity to small bacteriocin and repression of small bacteriocin production are located very close to the genes coding for medium bacteriocin production (Downie, personal communication; This study). pBAC11 is a large (30-40 bp) cosmid which carries the genes for bacteriocin production from 248 (Oresnik *et al.* 1999). Unlike 336, 336/pBAC11 was found not to inhibit 248 (Table 17, Appendix C). 336pBAC11 was also sensitive to bacteriocin production from 336, indicating that this cosmid contains the genes coding for small sensitivity and repression of small bacteriocin production.

Alternatively, the genes coding for the third bacteriocin produced by 306 could be encoded on more than one plasmid, or on one plasmid and the chromosome. The *nodO* gene, for instance, is encoded on the symbiotic plasmid pRl1JI, while its transporter genes, *prsDE,* are encoded elsewhere (Scheu *et al.* 1992). In 306, the separation of self-protection and toxin production genes could be a method of plasmid maintenance.

The bacteriocin encoded on pRle306c is a novel bacteriocin with unique properties which distinguish it from other rhizobiocins. For example, unlike the bacteriocin produced by 248, the novel 306 bacteriocin is much less stable in SF extracts, even when stored at 4 °C. The novel 306 bacteriocin is quickly inactivated at 45 °C, while the 248 bacteriocin is active at this temperature. Buffer and calcium treatments of bacteriocin-containing SF also affect the activity of the two proteins differently. Both bacteriocins show some calcium dependence, but the 248 bacteriocin is active at higher concentrations

of calcium (50mM) than the novel 306 bacteriocin (5mM). In addition, HEPES and PBS treatments of the 248 bacteriocin reduce activity, while the same treatments of the novel 306 bacteriocin do not affect bacteriocin activity. This physical characterisation of the 306 bacteriocin, although preliminary, establishes the bacteriocin as a previously uncharacterised rhizobiocin.

6.2 Characterisation of the Bacteriocin Encoding Region

Two thousand Tn*5* mutants of 306SM were screened for loss of bacteriocin activity, and two mutants were detected with altered activity against 248. M1 has no bacteriocin activity against 248, but appears phenotypically normal otherwise. The region of insertion of Tn*5* in M1 would therefore be expected to be close to or inside the region coding for bacteriocin activity against 248. M2 has only weak bacteriocin activity against 248 compared to 306SM. M2 also grows more slowly on TY plates than 306SM. It is possible that the location of the Tn*5* insertion in M2 is upstream of the bacteriocin encoding region, and that M2's reduced bacteriocin activity is due to polar effects of the transposon or the insertion of Tn*5* in a gene essential for the growth of 306SM (e.g. a housekeeping gene). However, there is no evidence based on sequence analysis to suggest that the Tn*5* insertion in M2 is directly inserted in a housekeeping gene.

The Tn*5*-containing regions from M1 and M2 were cloned out as *Eco*RI fragments and, for M1, as a larger *Kpn*I fragment. BLASTX sequence analysis of pAVM2 revealed limited homologies to Apx IV var 1, 2 and 3 from *A. pleuropneumoniae*, FrpA/C from *N. meningitidis*, and the rhizobiocin RzcA from 162Y10 (Schaller *et al.* 1999; Tettelin *et al.* 2000; Twelker *et al.* unpublished). BLASTX search results for pAVM1 also detected homologies to these proteins. This evidence suggests that the *Eco*RI fragments from M1 and M2 may be contiguous. BLASTX analysis of sequence data from pAVM3 detected low scoring homologies to TfuA, a potential housekeeping factor necessary for the

production of TFX (Breil *et al.* 1996). If pAVM1 and pAVM2 contain contiguous *Eco*RI fragments, pAVM3, which contains the *Eco*RI insert from pAVM1, should overlap both (Figures 13, 14, and 15). If this is the case, the Tn*5* insertion in M2 might affect downstream *tfu*A-like genes, resulting in slow growth and a bacteriocin-weak phenotype.

Medium bacteriocins from *R. leguminosarum* bv. viciae 248 and bv. trifolii 162Y10 have notable similarities to RTX toxins (Oresnik *et al.* 1999; Twelker *et al.* unpublished). The bacteriocin produced by 248 shows calcium dependence, and the predicted protein sequence has homologies to the calcium binding motif from RTX toxins (Oresnik *et al.* 1999). The predicted protein sequence from the bacteriocin encoding region of 162Y10 also has homologies to RTX toxins and associated ABC transporters (Twelker *et al.* 1999; Accession number AF141932). Medium bacteriocins of other *Rhizobium* strains might also be expected to have similarities to RTX toxins. The similarities of parts of the 306 bacteriocin encoding region to RTX toxins were not altogether unexpected.

The region following the sequence from pAVM1 showing homology to Apx IV var 3 contains one complete open reading frame (ORF2), and one partial open reading frame (ORF3). ORF2 and ORF3 are located almost 5 kb downstream of Tn*5* in M1. ORF2 and ORF3 are significantly homologous to HlyB-like ABC transporters and HlyD-like secretion proteins respectively. ORF3 also has less significant similarities to the *prsE* gene products from *R. leguminosarum* bv. trifolii and *S. meliloti*. PrsE is one of the proteins required for the secretion of NodO, which has similarities to RTX toxins (Finnie *et al.* 1997).

For subclones of pAVM1, sequence data obtained from the IS50 primers on Tn*5* revealed no significant homologies to medium bacteriocins or RTX toxins. Homologies to S-layer proteins, which are part of the RTX toxin family, and an RTX protein from

A. salmonicida were detected, but the homology scores based on BLASTX analysis were very low. However, RTX toxins are large (some are over 500 kDa). The predicted length of the bacteriocin from 162Y10 is over 4000 amino acids (Accession number AF141932). In addition, although the predicted protein sequence for the 162Y10 bacteriocin contains the RTX toxin motif and other regions with homologies to RTX toxins, there are also long stretches of DNA within the open reading frame with no significant homologies to any genes in the GenBank database (Hynes, personal communication). The predicted protein sequences for the 162Y10 bacteriocin and 248 bacteriocin share only limited homologies. Therefore, the absence of distinctive or extensive sequence homologies to RTX toxins or bacteriocins directly beside the Tn*5* insertion in M1 does not preclude its insertion in the structural gene coding for the bacteriocin from 306.

Given the extensive size (well over 11 kb) of the bacteriocin encoding region from 306SM, it is somewhat surprising that the frequency of the isolation of bacteriocin$^-$ mutants was so low (1/1000). In the environment, the frequency of spontaneous mutation in such a large gene region might be reduced in populations where selection pressures for bacteriocin production may exist. In the laboratory, these conditions would presumably be relaxed. It is possible that the transposon mutagenesis produced some insertions in the bacteriocin gene region which were not detected, since truncated proteins from 306 may also have bacteriocin activity. Transposon mutagenesis of the bacteriocin encoding region from 248 yielded many clones with bacteriocin producing phenotypes (Oresnik *et al.* 1999).

It would appear that the novel medium bacteriocin produced by 306 shares sequence similarities with RTX toxins and uses an ABC transporter system consistent with that used by other RTX toxins, such as haemolysin. As such, the bacteriocin transporter system likely includes an HlyB-like protein which localises to the inner membrane (Juranka *et al.* 1992). In addition, an accessory protein much like HlyD likely

aids in the translocation of the bacteriocin through the outer membrane. In the haemolysin model, *tolC*, which lies distal to the *hlyCABD* operon, is also required for secretion of HlyA (Wandersman and Delepelaire 1990). TolC is thought to span the outer membrane and thereby aid in the secretion of HlyA. To date, no *tolC*-like mutant has been found in 248, 162Y10 or 306 (Hynes, personal communication; This study). It is possible that *tolC*-like mutants of *Rhizobium* do not survive, although it is perhaps more likely that this specific outer-membrane factor is not required for bacteriocin secretion. Potentially, similar genes could be reiterated elsewhere in the genome.

To confirm that the ABC transporter and accessory protein sequenced from M1 were indeed responsible for the secretion of the novel bacteriocin, an attempt was made to create a 306 transporter mutant. An Ωspc cassette was introduced into a unique *Eco*RI site in the *hlyD*-like gene. The mutation was introduced into 306SM and 306DM using a gene replacement vector. Putative transporter mutants were screened for loss of activity against 248. Several 306SM mutants were found that had no activity against 248 in spot tests. Stab tests were inconclusive, as faint halos were produced, but reduced levels of secretion in the transporter mutants may still be possible via other mechanisms, and dead cells might also release the rhizobiocin. Furthermore, truncated HlyD mutants are still able to localise HlyA to the outer membrane, although HlyA is not excreted (Oropeza-Wekerle *et al.* 1990). The HlyD mutants in Oropeza-Wekerle *et al.*'s study did not have haemolytic activity. If in the case of the bacteriocin transporter mutants, the toxin was located in the outer membrane, it could be exposed enough to have partial activity against 248. The mechanism of action of HlyA is thought to be via pore formation in the membranes of eukaryotic cells (Benz *et al.* 1989). It has not been proven that rhizobiocins form pores in prokaryotic membranes, so complete excretion of the bacteriocin may not be necessary for partial activity. In addition, the bacteriocin could be excreted via an alternative secretion system. Despite certain amounts of specificity for

the regular transporter, RTX toxins from various species can be transported via the haemolysin transporter of *E. coli*. LktA, Cya and NodO can all be exported via the haemolysin system in *E. coli* (Highlander *et al.* 1989; Masure *et al.* 1990; Scheu *et al.* 1992). Since 306 produces three or more bacteriocins it seems possible that a different bacteriocin transport system could take-over, albeit inefficiently, the secretion of the novel bacteriocin in the putative transporter mutants.

The mechanism of self-protection against the novel 306 bacteriocin is unclear. The genes for bacteriocin production from 248 are encoded on pBAC12, and this clone appears to confer bacteriocin resistance to sensitive strains. For example, 336/pBAC12 is resistant to 248. However, the bacteriocin encoding region from 306 cloned in pAVRKM1 is not sufficient to confer bacteriocin resistance against 306 to 248. 248 clones carrying this region on 248pAVRKM1 grow very poorly in broth culture. But since this plasmid appears to carry a Tn*5* insertion in the bacteriocin region, active bacteriocin should not be produced from this plasmid. Potentially, the genetic load from this large plasmid causes 248 to grow more slowly. Self-protection against colicins is mediated by immunity proteins, but there is no conclusive evidence of an immunity protein in 162Y10, 248 or 306 (Oresnik *et al.* 1999; This study). Some lantibiotic producers appear to use the ABC transport machinery associated with their bacteriocins as a mechanism of self-protection (Sahl and Bierbaum 1998). It is hypothesised that any bacteriocin which has inserted into the membrane is transported back into the culture medium by the ABC transporters (Sahl and Bierbaum 1998). If such a system were present in 306, transporter mutants could be expected to be quite sickly. The putative transporter mutants in this study grew well on TY plates and in broth cultures, although less well on antibiotic plates.

6.3 The Role of Bacteriocin Production in the Competition for Nodulation

In broth culture, 306SM easily out-competes 248. However, the bacteriocin mutants are equally competitive, so bacteriocin production by 306 is unlikely to be related to its ability to out-compete 248 in broth. In addition, competition between strains cultured in broth is unlikely to mimic the conditions faced by rhizobia in the soil. From an agronomic stand point, competition for nodule occupancy is a more relevant measure of a strain's capacity for ecological success. Based on the results of the plant experiments, bacteriocin production by 306 does not appear to play a significant role in competition for nodulation between 306 and 248. This differs from findings from plant competition experiments comparing the competitive abilities of 248 and a bacteriocin mutant of 248 (Oresnik *et al.* 1999). In that study, bacteriocin production by 248 was found to play a role in competition between 248 and VF39, but not between 248 and the small bacteriocin producer, 3841. 3841 was highly successful at competing for nodulation even against 248 wild type. In the current study, 3841 was also very competitive. Strangely, the bacteriocin$^-$ 306 mutant was significantly better at competing with 3841 than the bacteriocin producing 306 parent strain, based on χ^2 analysis. No difference was expected, since the 306 and the mutant inhibit 3841 in bacteriocin assays to the same extent. A lack of random sampling from some of the plants may have skewed the results in this case.

Clearly many factors are involved in the competition for nodulation between rhizobia (Sadowsky and Graham 1998; Streeter 1994; Triplett and Sadowsky 1992). For example, Fix$^-$ strains are very poor competitors (George and Robert 1991; This study). Results from the first plant competition experiment in the current study were discarded due to the Fix$^-$ phenotype of 306SM and the mutants, since the plants appeared to select the Fix$^+$ symbionts over the Fix$^-$ strains. In the second plant competition experiment, 3841 and 248 were found to form nodules at a faster rate than 306DM and 306DM::Tn*5*.

The speed of nodule formation by 248 and 3841 was probably a major factor in their success at out-competing 306DM and the mutant. This is supported by other studies which indicate that speed of nodulation is important in competition (Malek *et al.* 1998: Stephens and Cooper 1988).

Bacteriocin production by 306 may not always play a role in competition for nodulation. Instead, it is possible that bacteriocin production is related to the interaction of 306 with the host plant. No studies have been conducted to assess the effect of rhizobiocins on eukaryotic cells. NodO, which has homologies to RTX toxins and the bacteriocin produced by 248, is capable of forming pores in lipid bilayers (Economou *et al.* 1990; Oresnik *et al.* 1999; Sutton *et al.* 1994). It has been suggested that NodO may mediate plant-rhizobia signalling by forming holes in the plant cell plasma membrane (Sutton *et al.* 1994). It is tempting to speculate that the homologies between rhizobiocins and RTX toxins suggest a role for rhizobiocins in plant-rhizobia interactions, perhaps in the initiation of the infection thread or in nodule formation. However, there is no evidence for this since bacteriocin$^-$ mutants and wild type strains are both able to nodulate plants (Oresnik *et al.* 1999; This study).

Other possible roles of rhizobiocins could involve functioning as a type of plasmid addiction molecule. This is supported by the fact that no pRle306c-cured derivatives of 306 have yet been obtained, although it is also possible that another gene may code for the addiction system in this strain (Hynes, personal communication). This possibility could be further explored by measuring the stability of plasmids carrying wild type and mutated bacteriocin genes.

A last, but remote possibility is that rhizobiocins such as those produced by 248, 162Y10 and 306 could have a structural role in the bacteria. The predicted proteins have limited homology to S-layer proteins of *Caulobacter* and *Campylobacter*, which are also members of the RTX family of proteins. The low levels of expression of rhizobiocin

genes do not, however, support such a role (Oresnik *et al.* 1999; Twelker, Oresnik and Hynes, personal communication).

6.4 Conclusions

R. leguminosarum bv. viciae strain 306 produces three bacteriocins with different activities. Genetic characterisation of the bacteriocin encoding region for one of these bacteriocins reveals significant homologies to RTX toxins and an accompanying ABC secretion system. The production of this novel bacteriocin by 306 does not appear to play a significant role in competition for nodulation under the conditions assayed. This argues against the potential use of the rhizobiocin gene from pRle306c in any attempt to genetically engineer more competitive strains of rhizobia.

6.5 Future Directions

The results of the current study open many possible avenues for continued investigation. Further study of the novel bacteriocin produced by 306 is in order, as well the role of this bacteriocin in competition and plant-rhizobial interactions.

Sequencing of the clones containing the bacteriocin encoding region from 306 has not been completed. Sequence analysis of the larger clones may be very revealing, and it would be interesting to find out if further homologies to RTX toxins or the characterised rhizobiocins exist. Sequence analysis may reveal a possible immunity protein. If not, bacteriocin resistant 248 isolates could be characterised in order to investigate the mechanism of resistance. Potentially, resistant mutants might have altered cell surface components which would otherwise serve as bacteriocin receptors.

Purification of the novel 306 bacteriocin should be carried out in order to conduct a comprehensive physical characterisation of the protein. SDS-PAGE analysis and gel filtration may be used to estimate the size of the bacteriocin. The effect of various agents

on the activity of the protein could also be carried out. For example, inactivation of the protein with β-mercaptoethanol could reveal the presence of disulphide bonds.

The 306 bacteriocin transporter mutants should be more closely characterised. First, Southern detection should be used to confirm the insertion of the Ω cassette in the clones. Second, the regions flanking the cassette should be cloned out, sequenced and characterised. Sequence analysis of genes downstream from the transporter region could reveal genes for accessory transporter proteins. It would also be interesting to find out if the bacteriocin transporter is required for other additional transport functions by studying the phenotypes of the transporter mutants more closely.

Finally, the role of bacteriocin production by 306 should be more thoroughly investigated. Further plant competition experiments in which other variables are minimised should be conducted, as well as field studies, which would provide a more accurate picture of the role of bacteriocin production in the environment. Other cultivars of pea and other host plants, such as vetch, could be included. And even if bacteriocin production were to be a factor in the competition for nodulation, another burning question persists: Is the inhibitory activity of rhizobiocins against closely related organisms only a side effect? This question could be addressed by examining the effect of bacteriocins on plant cells.

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Appendix A

1. Growth Media and Commonly Used Solutions

1.1.1 Luria Bertani Broth

yeast extract	5.0 g
tryptone	10.0 g
NaCl	10.0 g
distilled or	
deionized water	1.0 L

For solid media, add 15.0 g agar per 1 L broth.

(Miller 1972)

1.1.2 Two Times LB

yeast extract	1.0 g
tryptone	2.0 g
NaCl	1.0 g
deionized or	
distilled water	100 mL

1.2 PH Medium for Broth Cultures

peptone	4.0 g
tryptone	0.5 g
yeast extract	0.5 g
$CaCl_2$	0.2 g
MgSO4	0.2 g
distilled or	
deionized water	1 L

(Hynes *et al.* 1985)

1.3.1 TY Medium for Broth Cultures

tryptone	5.0 g
yeast extract	3.0 g
$CaCl_2$	0.4-0.5 g*
distilled or deionized water	1 L

(Beringer 1974)

1.3.2 TY Medium for Plates

tryptone	5.0 g
yeast extract	3.0 g
$CaCl_2$	0.5 g*
agar	12.5 g**
distilled or deionized water	1 L

(Beringer 1974)

*For **Soft TY Plates**, or **TY Broth for Bacteriocin Production**, 0.736 g $CaCl_2$ was used (Oresnik *et al.* 1999).

For **Soft TY Plates, 6.0 g agar was used (Oresnik *et al.* 1999).

1.4 Vincent's Minimal Media

Solution A

KH_2PO_4	1 g
K_2HPO_4	1 g
KNO_3	0.6 g
agar(DIFCO)	12.5 g

distilled or	
deionized water	1 L

Solution B

$FeCl_3$	0.1 g
$MgSO_4$	2.5 g
$CaCl_2$	1.0 g
distilled or	
deionized water	1 L

Solution C

biotin	0.01 g
thiamine	0.01 g
calcium pantothenate	0.01 g
distilled or	
deionized water	1 L

For 500 mL of medium, after autoclaving the solutions add together 450 mL A, 50 mL B, 5 mL C and 10 mL of the carbon source (20 % glucose, sucrose). For broth, the agar is not added to solution A.

(Vincent 1970)

1.5 YEM

mannitol	10 g
yeast extract	3 g
K_2HPO4	0.5 g
$MgSO_4*7H_2O$	0.2 g
NaCl	0.1 g
distilled or	
deionized water	1 L

(Vincent 1970)

For YEM supplemented with calcium, add 10 mL of $CaCl_2$ 50 mM per L.

1.6 Water Agar

agar	4.5 g
distilled or	
deionized water	300 mL

(Clark, personal communication)

1.7 Plant Growth Medium

Solution A

$CaCl_2$	294.0 g
distilled or	
deionized water	1 L

Solution B

KH_2PO_4	136.0 g
distilled or	

deionized water	1 L

Solution C

Fe-citrate	6.7 g
distilled or	
deionized water	1 L

Solution D

$MgSO_4$	123.0 g
K_2SO_4	87 g
$MnSO_4$	0.338 g
H_2BO_4	0.274 g
$ZnSO_4$	0.288 g
$CuSO_4$	0.1 g
$CoSO_4$	0.056 g
Na_2MoO_4	0.048 g
distilled or	
deionized water	1 L

1.0 mL of each solution is added to 2.0 L of distilled or deionized water, which is enough to fill six pots.

(Vincent 1970)

1.8 Phosphate-Buffered Saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
distilled or	
deionized water	up to 1 L

pH 7.34

(Sambrook, *et al.* 1989)

1.9 Tris Buffer (TBE)

EDTA	14.86 g
Tris Base	216.0 g
Boric Acid	110.0 g
deionized water	up to 20 L

Table 17. Concentration of Antibiotics Used in Growth Media (μg/ml)

Antibiotic	Bacteria/Media					
	E. coli		*Rhizobium*		*Agrobacterium*	
	broth	solid	broth	solid	broth	solid
Amp	50	100	NA	NA	NA	NA
Neo	NA	NA	50	100	NA	NA
Tet	2	10	2	5	NA	NA
Cm	20	20-30	NA	NA	NA	NA
Km	25	50	NA	10	NA	NA
Gm	10	15	15	30	15	50
Rf	NA	NA	NA	100	NA	100
Sm	NA	200	NA	600	NA	NA

NA- not applicable

Appendix B:

Growth Curves Demonstrating the Effect of Supernatant Fluid from 248 on the Growth of 336

In work related to the study of bacteriocin production by *R. leguminosarum* bv. viciae strain 306, a number of experiments were conducted in order to examine bacteriocin production from strain 248. In one set of experiments, growth curves were used to determine the effect of bacteriocin containing SF from 248 on a sensitive indicator strain, 336, in broth cultures. These experiments demonstrated that active bacteriocin was retained in 248 SF, and that activity was unaffected by filtering the SF with acrodiscs (Figure 24). In addition, bacteriocin activity was retained in chloroform treated SF (Figure 25). These data were considered in the design of expeiments to test for bacteriocin production from 306.

Figure 24. The Effect of Supernatant Fluid from 248 on the Growth of 336. A growth curve was conducted for 336 grown in the presence of SF from 336, 248, and the bacteriocin$^-$ mutant 248/pJB5JI. SF from 248 inhibited the growth of 336. SF from 248/pJB5JI inhibited the growth of 336, but to a lesser extent than 248 SF. Filtering the SF samples with low protein-binding acrodiscs was found to have no effect on bacteriocin activity.

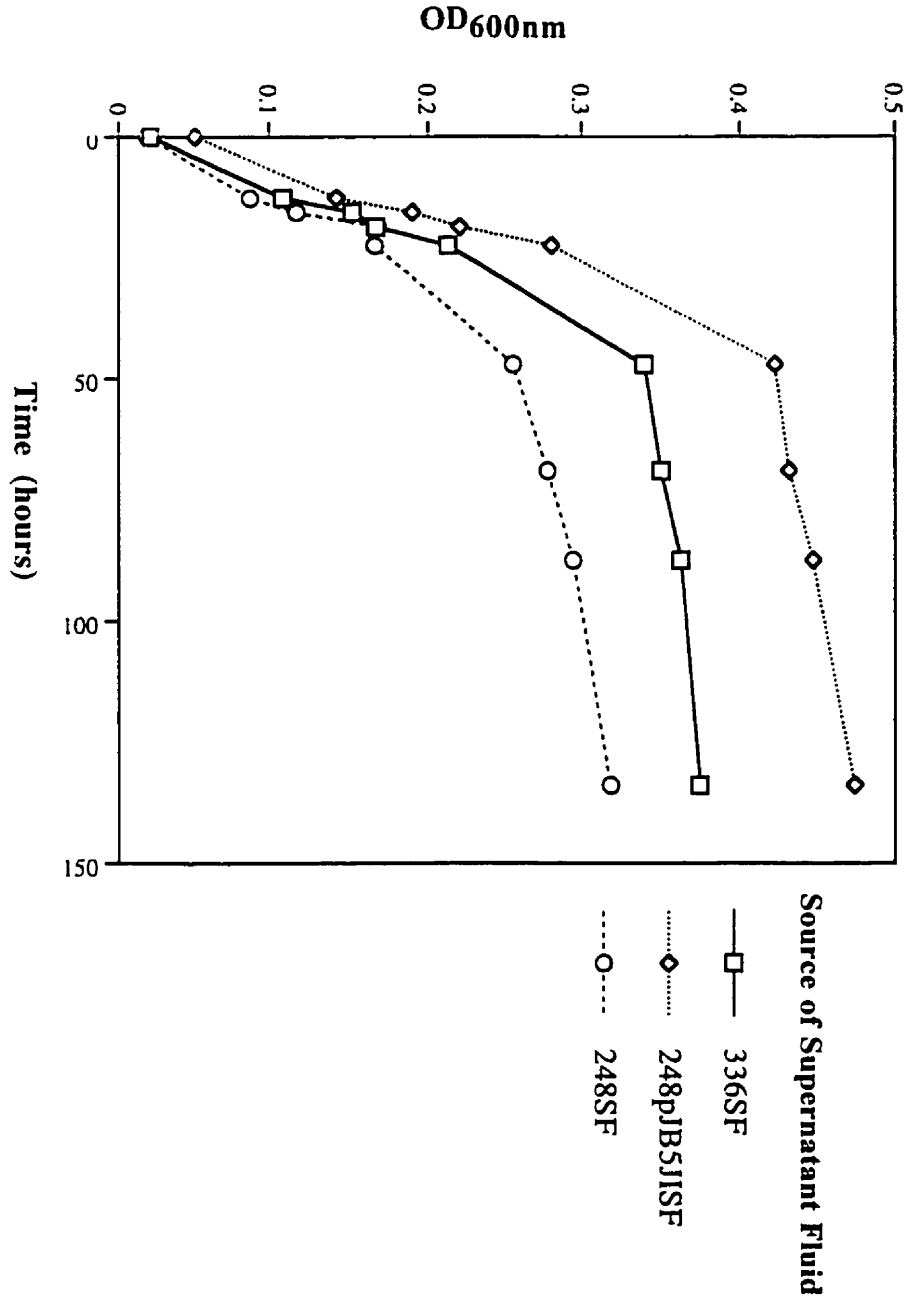
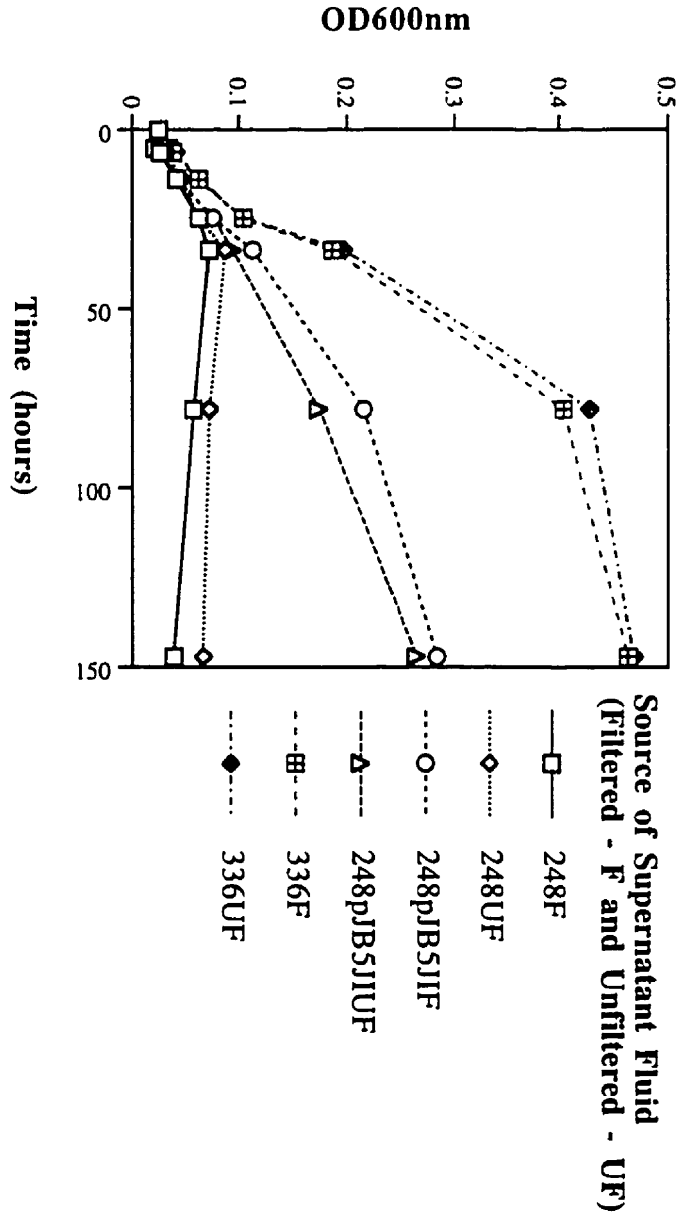

OD600nm
0
0.1
0.2
0.3
0.4
0.5
0
50
100
150
Time (hours)
Source of Supernatant Fluid
336SF
248pJB5JISF
248SF

Figure 25. The Effect of Chloroform Treated Supernatant Fluid from 248 on the Growth of 336. SF from 336, 248 and 248/pJB5JI was chloroform treated to remove potential chloroform soluble growth inhibitors. A growth curve was then conducted for 336 grown in the presence of the treated SF. Treated 248 SF inhibited the growth of 336 more than the control (336SF). Treated SF from the bacteriocin$^-$ mutant 248/pJB5JI also inhibited the growth of 336, but to a lesser extent than the bacteriocin producing wild type strain. In a subsequent growth curve, the chloroform treatment of 248 and 248/pJB5JI SF was found to have no effect on the inhibitory activity of the SF against 336.


OD600nm
0
0.1
0.2
0.3
0.4
0.5
0
50
100
150
Time (hours)
Source of Supernatant Fluid
(Filtered - F and Unfiltered - UF)
248F
248UF
248pJB5JIF
248pJB5JIUF
336F
336UF

Appendix C:

Bacteriocin Production and Resistance in 336 Clones Carrying Bacteriocin Gene Region from 248

Table 18. Bacteriocin Production and Resistance in 336 Clones

Indicator Strains	Strains Tested for Bacteriocin Production		
	248	336	336pBac11
248	-	+	-
336	+	-	?
336pBac12	W	-	NT
336pBac11	-	+	NT

- no inhibition, W weak inhibition, ? Inconclusive result, NT not tested, + inhibition

Stab tests were used to assay bacteriocin production/resistance.