BIOLOGICAL AND CHEMICAL ASPECTS OF AGROCIN 434 AS A SUPPLEMENTARY BIOCONTROL AGENT FOR CROWN GALL

NORMA N. FAJARDO

M.Sc. (University of the Philippines at Los Baños)

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Agricultural and Natural Resource Sciences at the University of Adelaide

Departments of Crop Protection and Plant Science

Waite Campus

The University of Adelaide

May 1995

TABLE OF CONTENTS

SUMMARY

The involvement of factors other than agrocin 84 in the biological control of crown gall by *Agrobacterium* strains K84 and K1026 has been suggested in a number of studies. This thesis examines the possible role in the biological control process of an antibiotic compound, agrocin 434, produced by the biocontrol strains in addition to the well-characterized adenine nucleotide agrocin 84.

A total of 66 *Agrobacterium* isolates from Australia, Europe and North America were tested for sensitivity to agrocin 434 using standard agrocin bioassay methods. All strains of biovar 2 tested were sensitive to agrocin 434 with the exception of 6 strains which produced agrocin 434 themselves. These included K84 and two agrocin 434-producing derivatives plus three isolates from New South Wales which were subsequently shown to be pathogenic. In contrast, all strains of biovars 1, 3 and *A. rubi* tested were resistant to agrocin 434. Selected isolates representing these three groups were also tested for agrocin 434 production to check if the resistance could be due to the production of this agrocin. None of those strains produced agrocin 434. It appears that sensitivity to agrocin 434 is a general characteristic of biovar 2 agrobacteria and may, at least in part, be chromosomally encoded.

It was also shown that media had an influence on the expression of sensitivity to agrocin 434. Whether this is due to inactivation of the agrocin, or to changes in the test strains brought about by growing on a particular medium remains to be clarified.

The genes involved in the biosynthesis of agrocin 434 are encoded on a large plasmid (300-400 kb) carried by K84 and some of its derivatives (Donner *et al.*, 1993). A derivative of K84 (strain K1143) which carries only this large plasmid, designated

pAgK434, has been isolated (Donner *et al.*, 1993). A buffered glucose-glutamate medium was found to support both the growth of K1143 and agrocin 434 production. Agrocin 434 was produced throughout the growth cycle even though its antibiotic activity is characteristic of a secondary metabolite. Agrocin production did not appear to be regulated by glucose.

Another derivative of K84, strain K1318, carries a deleted version of pAgK434 (the designation pAgK1318 is recommended). This strain produces a biologically inactive fragment of agrocin 434, designated nucleoside 4176, suggesting that part of the agrocin 434 biosynthetic pathway is encoded on pAgK1318 and that genes for this would most probably be located in the vicinity of the deletion. The smaller size of pAgK1318 (80-90 kb) facilitates the localization of genes involved in agrocin 434 synthesis as compared to the much larger pAgK434. On the assumption that the relevant genes are clustered in a manner similar to the agrocin 84 synthesis genes, mapping of pAgK1318 was undertaken. Conditions were optimized for the preparation and purification of plasmid DNA from strain K1318. A protocol for the minipreparation of plasmid DNA by the alkaline lysis method was scaled up and DNA was recovered from low melting point agarose gels using the enzyme agarase. The procedure is simple, fast and very reliable, yielding high quality DNA useful for most recombinant DNA applications.

*Xba*I digests of plasmids pAgK1318 and pAgK434 were compared and the results supported the assumption that a single large deletion event had given rise to plasmid pAgK1318 (McClure NC, unpublished). A novel 10.5 kb *Xba*I fragment of pAgK1318 encompasses the site of deletion and could be expected to contain some of the genes that encode the production of the biologically inactive agrocin 434 fragment. A physical map of the region inclusive of, and flanking, the novel 10.5 kb *Xba*I fragment was constructed with respect to the restriction endonucleases *Bam*HI, *Hin*dIII and *Sma*I

using the techniques of molecular cloning, DNA-DNA hybridization, and multiple and partial endonuclease digestions.

A provisional structure had been proposed for agrocin 434 as a disubstituted cytidine nucleoside (Donner SC, unpublished). The substituent at the N^4-position of cytidine was described as a β-1,4-hexuronosyl-glucopyranosyl moiety. The nature of the uronic acid component and its linkage remained to be established. Strong acid hydrolysis of agrocin 434 yielded glucuronic acid and glucose by electrophoretic comparisons with authentic samples. Sodium borohydride reduction of the acid hydrolysate yielded products whose electrophoretic properties in alkaline, as well as in borate and zinc complexing buffers, were identical with the reduction products of glucuronic acid and glucose. NMR studies have confirmed the nature of the linkages. The available evidence is consistent with a β-1,4-glucuronopyranosyl-α-1,N^4-glucopyranosyl substituent on the cytidine moiety of the nucleoside.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Norma N. Fajardo

May 1995

ACKNOWLEDGMENTS

I am deeply grateful to my supervisors, Dr. Max E. Tate, Dr. Nicholas C. McClure and Prof. Otto Schmidt, for their guidance, encouragement and support throughout the course of this study. I also wish to thank Dr. Bruce G. Clare, my former supervisor, for his encouragement and continued keen interest in my work in spite of his retirement.

I am grateful to Dr. David Liljegren for his help with university matters.

I would also like to thank Dr. Peter J. Murphy and Scott Donner for helpful discussions, Dr. Graham Jones for advice on cystallization and Dr. Kith Jayasena for assistance during manuscript preparation.

I sincerely thank the former members of Bruce Clare's lab, Garry Rosewarne and Natalie Dillon, for their help and friendship.

For assistance with laboratory and departmental matters, I am indebted to Terry Feckner, Gary Taylor, Anke Johnsen, and Emma Cabot.

I wish to thank Dr. Bob Brownlee of La Trobe University for NMR analysis of agrocin samples.

I thank the many friends I have made in the Departments of Crop Protection and Plant Science for their invaluable support and the warmth of their company.

Financial support from AusAid is gratefully acknowledged. I also thank the government of the Republic of the Philippines and, in particular, the University of the Philippines for giving me the opportunity to study in Australia.

Finally, I wish to express my deepest gratitude to my family for their love and support. I especially thank my husband, Freddie, and our four children, Brian, Marvin, Yasmin and Alvin. Their love has inspired and sustained me through the most difficult times in my study.

ABBREVIATIONS

Amp	Ampicillin
cm	centimeter
DNase	deoxyribonuclease
DIG	digoxigenin
FW	formula weight
g	gram
HVPE	high voltage paper electrophoresis
IR	infrared
Km	kanamycin
kb	kilobase
kV	kilovolt
l	lambda
l	liter
mg	microgram
ml	microliter
mm	micrometer
mg	milligram
ml	milliliter
mM	millimolar
min	minute
M	molar
nm	nanometer
NMR	nuclear magnetic resonance
OD	optical density
RM	relative mobility
RNase	ribonuclease

SDS	sodium dodecyl sulfate
T-DNA	transferred-DNA
Ti	tumor-inducing
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
w/v	weight per unit volume

CHAPTER ONE

GENERAL INTRODUCTION

REVIEW OF LITERATURE

1.1 INTRODUCTION

Crown gall is a plant cancer which causes significant international losses to crops such as stone fruit, grapes, pome fruit, nuts, roses and other ornamentals (Schroth and Moller, 1976; Moore and Warren, 1979; Panagopoulos *et al.*, 1979; Alconero, 1980; Kennedy and Alcorn, 1980; Kerr, 1989). The disease is characterized by tumor formation at infected wound sites, usually at the crown (hence the name crown gall) or on the root system (Figure 1.1). It affects mainly dicotyledonous plants and a few gymnosperms (Lippincott and Lippincott, 1975; De Cleene and De Ley, 1976). Monocots appear to be naturally resistant but some have been shown to be susceptible following artificial inoculation (Hooykaas-Van Slogteren *et al.*, 1984; Graves and Goldman, 1986; Grimsley *et al.*, 1987).

Since 1907 when Smith and Townsend first reported the bacterial origin of this neoplastic disease of plants (Braun, 1982), a vast amount of research has been undertaken worldwide in order to understand the molecular mechanism of crown gall pathogenesis. The momentum for such research has been sustained by advances in molecular biology and genetic techniques which have revolutionized the understanding of the plant-bacterial interactions involved in crown gall induction. Key steps in the process are now known although there are many facets of the pathogenesis which are poorly understood.

Figure 1.1 Crown gall on almond induced by pathogenic strain K27. Arrows indicate galls. (Photo courtesy of Ali Reza Ahmadi)

A biological control system (Kerr, 1980; Kerr, 1989; Jones and Kerr, 1989; Farrand, 1990; Clare, 1993) developed against the disease has found acceptance in many countries around the world. This biological control system is based on the activities of a non-pathogenic *Agrobacterium* strain, K84. Evidence has gradually accrued (Cooksey and Moore, 1982; Shim *et al.*, 1987; Macrae *et al.*, 1988; Lopez *et al.*, 1989; Vicedo *et al.*, 1993; Farrand, 1990; Farrand and Wang, 1992) that the full range of mechanisms by which this extraordinarily successful agent inhibits pathogenesis still remains to be clarified. Examination of the possible role of factors other than agrocin 84 in the biocontrol system forms a central part of this thesis.

1.2 THE GENUS AGROBACTERIUM

Crown gall is caused by pathogenic strains of *Agrobacterium*, a soil microorganism which belongs to the family Rhizobiaceae along with *Rhizobium*, *Bradyrhizobium* and *Phyllobacterium* (Jordan, 1984). Members of the genus are Gram-negative, single-celled, non-sporeforming rods motile by means of a few peritrichous flagella (Kerr and Brisbane, 1983). Although all strains grow between 20 and 28°C, the optimum temperature for growth is 25-28°C.

The taxonomy of agrobacteria has been the subject of controversy and much of it stems from the use of species epithets that are closely associated with unstable pathogenic characteristics (Young *et al.*, 1992). For example, the names *A. tumefaciens* and *A. radiobacter* have been applied to strains which differ only in the presence or absence, respectively, of a tumor-inducing (Ti) plasmid which can be either lost or transferred between strains (Kerr, 1969; Van Larebeke *et al.*, 1975). Another example is the association of the names *A. tumefaciens* and *A. rhizogenes* with tumor-inducing and root-inducing capacity, respectively, again emphasizing transient pathogenic attributes. Even without such a distinction, these two are considered separate species on the basis of phenotypic characteristics and DNA relatedness (Holmes and Roberts, 1981; Kersters and De Ley, 1984). If names are considered without regard to their descriptive

connotations, there are four valid species, namely, *A. tumefaciens*, *A. rhizogenes*, *A. vitis* and *A. rubi* (Ophel and Kerr, 1990; Young *et al.*, 1992).

To avoid the confusion in the nomenclature of *Agrobacterium*, there has been a strong tendency to use biovar designations which are based on characteristics that are chromosomally determined (Kerr, 1992; Clare, 1995). Biovars 1, 2 and 3 correspond to *A. tumefaciens*, *A. rhizogenes* and *A. vitis*, respectively, while *A. rubi* has no biovar designation (Ophel and Kerr, 1990; Kerr, 1992). Agrobacteria can thus be referred to as biovar 1-3 strains or *A. rubi* without regard to their pathogenic characteristics. Recently, on the basis of 16S ribosomal RNA (rRNA) studies, Sawada *et al.* (1993) proposed the replacement of *A. tumefaciens* by *A. radiobacter* as the valid species epithet for biovar 1 strains.

In nature, agrobacteria have been found to have specific host associations (Ophel and Kerr, 1987). While biovar 1 strains have been associated with a wide range of dicotyledonous hosts, biovar 2 strains have mostly been found in stone fruit. Biovar 3 strains affect grapevines virtually exclusively while *A. rubi* have been isolated only from *Rubus* spp. (Ophel and Kerr, 1987; Kerr, 1992). However, many agrobacteria have been shown to have a wider host range in artificial inoculations (Ophel and Kerr, 1987).

1.3 THE ROLE OF PLASMIDS IN CROWN GALL INDUCTION

Crown gall is a neoplastic disease that results from the stable integration of a discrete segment of a large plasmid (the tumor-inducing or Ti-plasmid) into the nuclear genome of the infected plant. It was several decades after the bacterial nature of the disease was recognized before the Ti-plasmid was finally identified as the tumor-inducing principle. A key discovery was made by Kerr (1969) who reported that virulence could be transferred to non-pathogenic isolates in a developing crown gall tumor suggesting the possibility that a transmissible extrachromosomal element determined pathogenicity. Zaenen *et al.* (1974) later found that virulent strains of agrobacteria carry one or more large plasmids and suggested that genetic information for

crown gall induction could reside on one or several of those plasmids. The role of the Ti-plasmid in crown gall induction was firmly established when Van Larebeke *et al.* (1975) showed that the acquisition of the large plasmid from pathogenic strains was the reason why non-pathogenic isolates became virulent in mixed infections *in planta*. It was subsequently demonstrated that only a portion of the Ti-plasmid was transferred from pathogenic agrobacteria to plant cells (Chilton *et al.*, 1977) where it is stably integrated into the host nucleus (Yadav *et al.*, 1980).

1.4 THE MOLECULAR MECHANISM OF PATHOGENESIS

Since the discovery of the relationship between Ti-plasmid and pathogenicity much work has gone into understanding the molecular mechanism that leads to the tumorigenic phenotype. This mechanism is now known in some detail and is the subject of several recent reviews (Winans, 1992; Zambryski, 1992; Clare and McClure, 1995).

Several discrete steps are involved in crown gall induction. The pathogenic process is initiated by bacterial recognition and attachment to susceptible wounded plant tissues. Then a sequence of events leads to the transfer of a specific segment of the Ti-plasmid (the transferred DNA or T-DNA) to the plant cell nucleus where it becomes covalently integrated. The genes on the T-DNA are expressed in the plant cells. As well as expressing functions which result in the tumorigenic growth, T-DNA genes also encode the synthesis of a novel group of compounds termed opines which not only provide the pathogen with an additional source of nutrients but also ensure the organism's spread and survival by inducing conjugal transfer of Ti-plasmids (Binns and Thomashow, 1988; Zambryski, 1988; Clare, 1990).

1.4.1 Chemotaxis and Attachment

The initial event in crown gall induction is a positive chemotactic response to plant exudates, mainly phenolic compounds and certain sugars, released by wounded plant tissue (Stachel *et al.*, 1985; Ashby *et al.*, 1987; Ashby *et al.*, 1988;). Two levels

of chemotaxis towards plant metabolites have been demonstrated, one chromosomally encoded and the other Ti-plasmid dependent (Ashby *et al.*, 1987; Parke *et al.*, 1987; Ashby *et al.*, 1988; Shaw *et al.*, 1988).

The next step in the process is attachment of the bacteria to the plant tissue, a process which necessarily involves bacterial cell-plant cell recognition. Little is known about plant components required for attachment but agrobacterial components have been identified. Three chromosomal virulence loci, *chvA* and *chvB* (Douglas *et al.*, 1985) and *pscA* (Thomashow *et al.*, 1987) have been shown to encode products required for bacterial attachment during the infection process. The *chvB* locus appears to be involved in the synthesis of a β-1,2-glucan which has been implicated in plant cell binding (Zorreguieta *et al.*, 1988), while *chvA* may encode a transport function for the polysaccharide (Cangelosi *et al.*, 1989). The *pscA* locus, shown to be related to the exoC locus of *Rhizobium meliloti* (Marks *et al.*, 1987), encodes a gene product which may have a role in the production of surface polysaccharides important for attachment to plant cells. Whether this role is a direct one or not remains to be clarified (Thomashow *et al.*, 1987).

Two other genetic loci have been implicated in bacterial attachment. The *cel* locus is important in cellulose biosynthesis (Robertson *et al.*, 1988) while the closely-linked *att* genes encode cell surface polypeptides which may play a role in bacterial binding to plant cells (Matthyse, 1987; Robertson *et al.*, 1988). Mutations in these loci lead to either loss or attenuation of virulence.

1.4.2 T-DNA Processing and Transfer

Mutational and transcriptional studies have confirmed that genes clustered in a 40-kb segment of the Ti-plasmid known as the *vir* region are responsible for the excision and transfer of T-DNA from *Agrobacterium* to the plant cell nucleus. The *vir* region comprises about 25 genes arranged in either 6 or 7 operons (*virA-virG*) which are co-regulated thus forming a regulon (Winans, 1992; Hooykaas and Schilperoort, 1992).

The majority of the *vir* genes are not expressed at detectable levels until they are induced, in contrast to the known chromosomal virulence genes which are constitutively expressed. The phenolic compounds acetosyringone and α-hydroxyacetosyringone were the first compounds to be identified as inducers of the *vir* regulon (Stachel *et al.*, 1985). In addition to these two, other compounds such as monosaccharides, their derivatives and analogues have subsequently been implicated in *vir* gene induction (Veluthambi *et al.*, 1989; Ankenbauer and Nester, 1990; Cangelosi *et al.*, 1990; Shimoda *et al.*, 1990).

The *vir* gene regulatory system operates through 2 monocistronic genes, *virA* and *virG*, which resemble genes of other two-component prokaryotic regulatory systems such as *envZ-ompR*, *ntrB-ntrC* and *dctB-dctD* (Nixon *et al.*, 1986; Parkinson and Kofoid, 1992). The constitutively expressed *virA* probably acts as a chemoreceptor for plant wound metabolites and transmits this signal to the bacteria by activation (probably by phosphorylation) of the VirG protein. VirG then activates the transcription of the other *vir* genes (Stachel and Zambryski, 1986; Jin *et al.*, 1990a ; Jin *et al.*, 1990b; Turk *et al.*, 1994). Products of the *virB*, *C*, *D* and *E* loci have been ascribed functions relating to the cleavage of T-DNA at the border sequences and its processing and transfer to the host. The *virH* (also known as *pinF*) is generally not essential for virulence but may be involved in the protection of the bacteria during the infection process (Zambryski, 1992).

Experiments have shown that the 25 bp imperfect direct repeats (LB and RB) flanking the T-DNA on the Ti-plasmid are important in the transfer process (Yadav *et al.*, 1982; Zambryski *et al.*, 1982). In a concerted action proteins encoded by *virD* recognize and cleave the bottom strand of the 25 bp border repeat sequence (Yanofsky *et al.*, 1986). The nicked borders are then used for the generation of T-strands, molecules considered to be the T-DNA intermediates that are transported from the bacteria into the plant (Stachel *et al.*, 1986). In later experiments, it was established that the right border alone is essential for the transfer of genes (Wang *et al.*, 1984). In octopine-type Ti-plasmids there is, in addition to the 25 bp border sequences, a specific 24 bp sequence called "overdrive" at 13-14 bp from the right border sequence which enhances T-DNA transfer (Peralta *et al.*, 1986; Van Haaren *et al.*, 1987). Evidence indicates that VirC1

can bind to the overdrive sequence and that this interaction enhances T-strand production (Toro *et al.*, 1989). There is no sequence that closely resembles the octopine "overdrive" near nopaline borders (Wang *et al.*, 1987).

As the first step in T-strand transport is traversing the bacterial membrane, some *vir* functions must provide for alteration of the bacterial membrane to facilitate T-strand passage. Since most of the 11 *virB* open reading frames have sequences characteristic of genes encoding either transmembrane or membrane-associated proteins, VirB proteins are thought to form a structure (pore or channel) through which the DNA is delivered into the plant cell (Kuldau *et al.*, 1990; Shirasu *et al.*, 1990).

The VirE proteins are likely candidates as components of the T-complex that is transported to the plant cell nucleus. This is consistent with their being the most abundant products of *vir* induction (Citovsky *et al.*, 1988; Zambryski, 1992) and the finding that the VirE2 polypeptide binds single-stranded DNA (Christie *et al.*, 1988). Experimental evidence suggest that the transferred form of the T-DNA is single-stranded (Stachel *et al.*, 1986; Winans, 1992; Zambryski, 1992; Tinland *et al.*, 1994). However, double-stranded molecules may also be produced (Dürrenberger *et al.*, 1989) and could possibly play a role in the natural transformation process (Tinland *et al.*, 1994).

1.4.3 T-DNA Integration and Expression

Integration of the T-DNA into the plant nuclear genome results in the expression of oncogenicity (*onc*) genes which code for the production of phytohormones. The genes *iaaM* and *iaaH* encode enzymes, a monooxygenase and a hydrolase, which together catalyze the conversion of tryptophan via indoleacetamide (IAM) into indole acetic acid (IAA), a compound with auxin activity.(Thomashow *et al.*, 1984; Thomashow *et al.*, 1986; Schroder *et al.*, 1984). The *ipt* gene codes for an isopentenyl transferase, an enzyme which catalyzes the conversion of AMP into isopentenyl-AMP, a compound with cytokinin activity (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984). Elevated

levels of these phytohormones give rise to uncontrolled cell proliferation and tumor formation (Van Onckelen *et al.*, 1984).

As well as expressing the *onc* genes, the T-DNA also directs the synthesis (Holsters *et. al.*, 1980) of one or more of a novel group of amino acid derivatives collectively called opines (Tempé and Goldman, 1982). The opines induce conjugal transfer of Ti-plasmids among agrobacteria and also encode gene products required for opine catabolism (Dessaux *et al.*, 1992; Dessaux *et al.*, 1993). Even before the discovery of Ti-plasmids and T-DNA it had already been hypothesized, based on early studies on opines, that the transfer of a portion of the bacterial genome into the plant cell could be responsible for crown gall. The hypothesis was confirmed with the advent of recombinant DNA techniques which lent genetic support for the hypothesis (Dessaux *et al.*, 1992). That opines play a fundamental role in *Agrobacterium*-plant interactions – that of "chemical mediators of parasitism"– is embodied in what is generally known as the Opine Concept (Tempé and Petit, 1983). In this concept the bacteria sequester the plant metabolic machinery for the production of compounds that will ensure their survival and spread. The mechanism by which they achieve this is by genetic engineering. J. Schell and his associates termed the process genetic colonization (Tempé and Petit, 1983). While initially it was thought that only the inciting bacteria are capable of utilizing opines, later investigations have shown that microorganisms such as fungi and other types of bacteria including pseudomonads and coryneform bacteria are also able to catabolize opines (Beauchamp *et al.*, 1990; Nautiyal and Dion, 1990).

In contrast to the vast amount of information that has been generated on the agrobacterial aspect of DNA transfer, little is known about the process of DNA integration in the host plant. Willmitzer *et al.* (1980) localized the position of the T-DNA in the nucleus of the transformed plant cell. The expression of bacterial genes in a system that is known to recognize a different set of expression signals remained a mystery for some time. That the T-DNA has eukaryotic properties was first confirmed by experiments wherein transcription of T-DNA was shown to be inhibited by α-amanitin, an inhibitor of RNA polymerase II, suggesting that expression of T-DNA

genes depends upon this RNA polymerase (Willmitzer *et al.*, 1981). Subsequently, the complete T-DNA sequence of the octopine Ti-plasmid was reported (Barker *et al.*, 1983) and made possible a detailed analysis of the 5' and 3' non-coding regions of the T-DNA. Indeed, eukaryotic expression signals such as the TATA box for transcription initiation and the AATAAA box for transcription termination and polyadenylation were identified. However, introns, a feature of many eukaryotic genes, were not found.

The transfer of DNA from *Agrobacterium* to the plant cell has been likened to the bacterial conjugation system. Several lines of evidence derived mainly from studies of incompatibility groups IncP1 and IncW plasmids support this analogy (Zambryski, 1992; Kado, 1993). The most direct support, however, comes from Buchanan-Wollaston *et al.*'s (1987) demonstration that the *oriT* of the conjugative bacterial plasmid pRSF1010 can direct DNA transfer to the plant in place of the T-DNA borders. It has been pointed out, though, that while the early stages of T-DNA processing and transport may resemble bacterial conjugation, the later steps, particularly those occurring inside the plant cell, share common features with viral infection (Zambryski, 1992).

1.5 BIOLOGICAL CONTROL OF CROWN GALL

1.5.1 The K84 System

The biological control of certain crown gall pathogens has been practiced on an international scale for the past twenty years and utilizes a naturally-occurring non-pathogenic biovar 2 agrobacterial strain, K84. The procedure that Kerr and associates developed involves dipping planting materials such as cuttings, seeds or seedlings in suspensions of K84 cells immediately before planting (New and Kerr, 1972; Htay and Kerr, 1974; Kerr, 1980).

Strain K84 was isolated from soil around an infected peach tree near Adelaide, South Australia early in the 1970's (New and Kerr, 1972). Since 1973, K84 has been sold commercially in Australia (Jones *et al.*, 1988) and successful biological

control of crown gall on several hosts has been reported from many countries around the world (Schroth and Moller, 1976; Matthee *et al.*, 1977; Moore, 1977; Moore, 1979; Moore and Warren, 1979; Panagopoulos *et al.*, 1979). It must be emphasized, however, that K84 is only preventive, not curative.

The remarkable success of K84 as a biological control agent stimulated much interest in elucidating the mechanism(s) by which the control process occurs. Kerr and Htay (1974) demonstrated that control is achieved through the production of a bacteriocin which they called bacteriocin 84 (later changed to agrocin 84). Bacteriocins are antibiotic compounds produced by certain strains of bacteria, which are inhibitory to other strains of the same or closely related species (Kerr, 1982). Prior to the discovery of the nucleotide bacteriocins, most definitions have emphasized the proteinaceous nature of these compounds. Engler *et al.* (1975) coined the name agrocin to refer to low molecular weight antibiotic substances produced by certain strains of *Agrobacterium*, which are inhibitory to other strains of agrobacteria. The marked specificity of agrocins against closely related strains is a characteristic that they share with the other bacteriocins.

The genes for the biosynthesis of agrocin 84 are located on a 47.7 kb plasmid called pAgK84 (Ellis *et al.*, 1979; Slota and Farrand, 1982) on which regions coding for plasmid transfer and immunity to inhibition by agrocin 84 have also been identified (Farrand *et al.*, 1985; Ryder *et al.*, 1987). Wang *et al.* (1994), through mutational and complementation analysis, have demonstrated that the 21-kb region on pAgK84 encoding agrocin 84 synthesis is organized into at least 5 transcriptional groups which they have named *agnA* through *agnE*. They have also shown that the *agn* genes are expressed *in planta* giving further support to the hypothesis that, in the soil, agrocin 84 production by strain K84 is important in the control of crown gall caused by sensitive strains.

Most of the studies on the genetics of sensitivity to agrocin 84 have been carried out on the nopaline Ti-plasmid pTiC58. The *acc* genes on this plasmid encode transport and catabolism of the opines agrocinopine A and B (Ellis and Murphy, 1981;

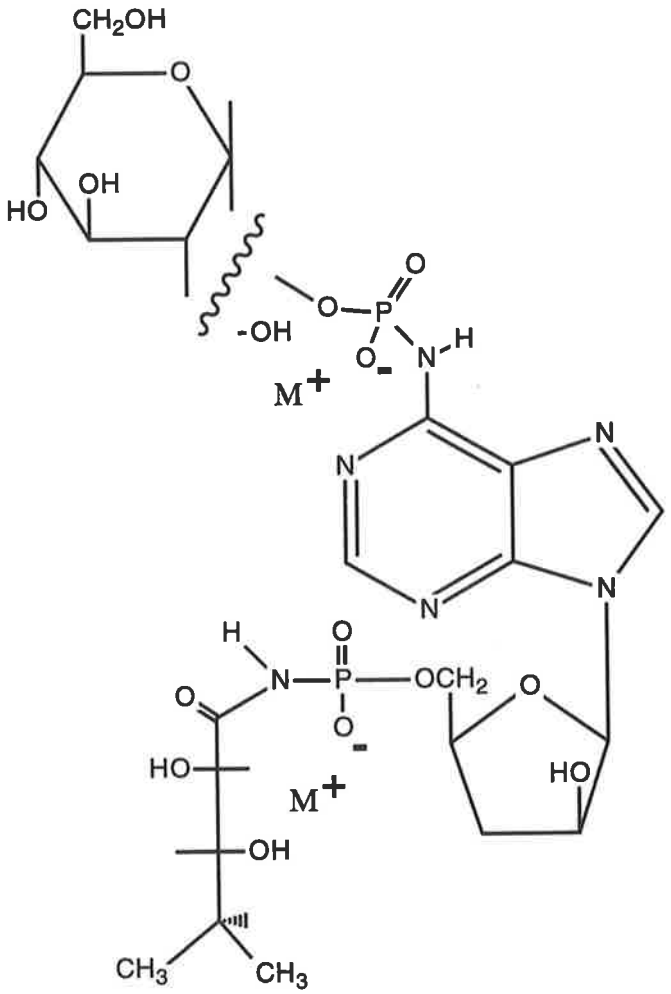
Ryder *et al.*, 1984; Hayman and Farrand, 1988; Hayman *et al.*, 1993). Transport of these opines involves a periplasmic permease which has also been shown to actively take up agrocin 84 due to its N^6-D-glucofuranosyloxyphosphoramidate substituent (Murphy and Roberts, 1979; Ellis and Murphy, 1981; Murphy *et al.*, 1981; Hayman and Farrand, 1988; Hayman *et al.*, 1993). Strains with *acc* genes are therefore sensitive to agrocin 84 – this is the basis for the extraordinary selectivity of agrocin 84 for pathogens carrying a nopaline Ti-plasmid.

Agrocin 84 (Figure 1.2) is a disubstituted adenine nucleotide analogue (Murphy *et al.*, 1981). From structure-function studies of the molecule, it was established that uptake of the agrocin was determined by the N^6-D-glucofuranosyloxyphosphoramidate substituent and that toxicity was conferred by the 5'-phosphoramidate moiety (Tate *et al.*, 1979; Murphy *et al.*, 1981).

Little is known about the exact mechanism of action of agrocin 84 in inhibiting cell growth but Kerr and Tate (1984) have pointed out the structural similarity of the lower (or alpha) face of the β-D-3'-deoxyarabinofuranosyl group of the agrocin 84 molecule to the dideoxynucleotides used in DNA sequencing reactions for chain termination. Murphy *et al.* (1981) have established that the presence of a fraudulent nucleotide moiety alone is not sufficient for growth inhibition and that the minimum moiety of agrocin 84 required for its toxic action is the 5'-phosphoryl substituted nucleoside. In studies using radiolabelled precursors, measurable effects on DNA synthesis have been observed (McCardell and Pootjes, 1976; Das *et al.*, 1978).

The continued success of K84 as a biological control agent was potentially threatened by the fact that pAgK84 could transfer conjugatively to tumorigenic strains which would then acquire immunity to agrocin 84 (Ellis and Kerr, 1979; Panagopoulos *et al.*, 1979; Farrand *et al.*, 1985). Recombinant DNA techniques were used to construct a deletion mutant of K84 which is unable to transfer its plasmid to other agrobacteria (Jones *et al.*, 1988). This genetically-engineered derivative, strain K1026, was shown to be as effective as K84 in preventing crown gall on almond seedlings in open air pot trials (Jones and Kerr, 1989). Strain K1026 was subsequently registered as the pesticide

Figure 1.2 Structure of agrocin 84, a 3'-deoxyarabinofuranosyladenine bisphosphoramidate.



AGROCIN 84

product Nogall™ and became the first genetically-engineered microbe to be released for commercial use (Kerr, 1989).

Another possible threat to the effectiveness of both K84 and K1026 is their acquisition of a Ti-plasmid which would make them agrocin 84 immune pathogens. This situation is considered highly unlikely because both biocontrol strains carry the nopaline catabolic plasmid pAtK84b which belongs to the same incompatibility group as the nopaline Ti-plasmids of the tumorigenic strains (Clare *et al.*, 1990). Plasmids belonging to the same incompatibility group cannot exist within a bacterial cell at the same time.

Agrocin production is conceivably not the sole means by which control of crown gall is effected by the biocontrol strains. It has been suggested that they are also able to colonize roots efficiently giving them competitive advantage over their pathogenic counterparts (Ellis *et al.*, 1979; Cooksey and Moore, 1982; Shim *et al.*, 1987; Macrae *et al.*, 1988; Lopez *et al.*, 1989; Vicedo *et al.*, 1993). However, Farrand and Wang (1992) pointed out that there is a need for a more critical assessment of the role of root colonization as the parameters involved are not clearly understood. They also argued that there is a lack of consensus even on the definition of colonization.

1.5.2 Other Potential Biological Control Agents

Both the success of K84 to control certain crown gall pathogens and its failure to control others have emphasized the importance of finding other possible biological antagonists. In Stonier's (1960) original paper on agrocins, he found that two strains, H100 and T37, were able to inhibit several other strains of agrobacteria and attributed this to the production of an antibiotic substance. Kerr and Htay (1974), during experiments conducted to demonstrate that K84 inhibits pathogenic strains through production of agrocin 84, found that a pathogenic strain, K108, produced its own agrocin. It was later shown that agrocin 108 is also a fraudulent nucleotide (Kerr and Tate, 1984).

In South Africa, Hendson *et al.* (1983) isolated strain D286, a pathogen that spontaneously lost its pathogenicity. The agrocin it produced was found to inhibit strains carrying nopaline-, octopine- and agropine-type Ti-plasmids. The physical characteristics of agrocin D286 were consistent with its being a nucleotide, like agrocin 84. In a comparative study of D286 and K84, Van Zyl *et al.* (1986) found that K84 was generally superior to D286 in preventing tumor formation. In addition, it was shown that no biotype 3 strains were inhibited by the two strains *in vitro*. Another agrocin-producing South African isolate, J73, inhibited grapevine strains. This find was particularly encouraging because of the known resistance of *A. vitis* pathogens to control by K84. However, J73 is a pathogen and its full potential can only be exploited once it is cured of its Ti-plasmid.

In China, non-pathogenic strains HLB2 (Chen and Xiang, 1986; Pu and Goodman, 1993) isolated from a hop gall, and E26 (Liang *et al.*, 1990) have been found to inhibit crown gall on grapevines.

The search for other antagonists has not been limited to agrobacteria. Cooksey and Moore (1980) investigated both the *in vitro* and *in vivo* inhibition of pathogenic agrobacteria using fungal and bacterial antagonists. In field tests, galling caused by some of the tumorigenic strains tested was reduced by isolates of *Penicillium*, *Aspergillus*, *Bacillus* and *Pseudomonas*. However, none was found to be more effective than K84.

So far, the full potential of these prospective biocontrol agents have not been adequately investigated. It will probably take some time before one can be found that will match the remarkable success of K84 in the field.

AIMS AND SCOPE OF STUDY

Inhibition by K84 of *Agrobacterium* strains resistant to agrocin 84 suggests that other mechanisms are important in the overall effectiveness of K84 in controlling crown gall. Indeed a second agrocin has been isolated which is produced by the biocontrol strains K84 and K1026 and some of their derivatives (Donner *et al.*, 1993). The agrocin was designated agrocin 434 after the strain from which it was first isolated, K434, a derivative of K84 which has lost the agrocin 84 plasmid, pAgK84.

Preliminary studies in this institute (Donner SC, unpublished) have suggested that this novel agrocin is inhibitory to biovar 2 agrobacteria. A more extensive survey was conducted in this study involving strains belonging to biovars 1, 2 and 3 and *A. rubi* in order to establish the selectivity properties of agrocin 434. The effect of media on sensitivity to this particular antibiotic was also investigated.

The largest of the K84 plasmids, previously known as the cryptic plasmid (300-400 kb), has been shown to encode functions involved in the biosynthesis of agrocin 434. The name pAgK434 was thus recommended. The biosynthetic genes need to be localized on this large plasmid. To this end it was deemed that one workable approach would be to use a derivative of K84, K1318, which carries a deleted version of pAgK434 and which produces a biologically inactive fragment of agrocin 434. Restriction endonuclease mapping of a region of pAgK1318 (the deleted plasmid) likely to contain some of the genes involved in agrocin 434 synthesis was undertaken.

Any investigation on mechanisms of control by antibiotic compounds must necessarily involve structure-activity relationships, hence the importance of knowing the chemical structure of compounds being studied. The structure of agrocin 434 as a disubstituted cytidine nucleoside has been partially elucidated (Donner SC, unpublished). A particular aim of the present study was to finally establish the nature of the N^4 aldobiouronic acid substituent of agrocin 434.

CHAPTER TWO

SCREENING AGROBACTERIA FOR INHIBITION BY AGROCIN 434

INTRODUCTION

The biological control of crown gall has been practiced on an international scale since 1973 (Kerr, 1989) using a non-pathogenic strain of *Agrobacterium*, strain K84. An improved derivative of K84, strain K1026, developed by recombinant DNA techniques (Jones *et al.*, 1988) has been approved for commercial release in Australia and is now being marketed as the pesticide product Nogall™ (Kerr, 1989).

Whilst the efficacy of K84 and K1026 as biocontrol agents has been attibuted to the inhibitory effect of agrocin 84 (Kerr and Htay, 1974; Kerr, 1980), it now seems likely that other mechanisms play a role in the biocontrol process. In field studies conducted by Lopez *et al.* (1989), both K84 and a mutant strain unable to produce agrocin 84 controlled strains resistant to agrocin 84 in culture. In greenhouse experiments, Cooksey and Moore (1982) were also able to reduce infection on tomato seedlings caused by an agrocin 84-resistant strain by using a mutant that no longer produced agrocin 84. They suggested that physical blockage of the infection site, among other factors, could be responsible for the results obtained. Farrand and Wang (1992) have also suggested that efficient root-colonizing ability and the production of other agrocins could be important in the overall effectiveness of the K84 system.

A second antibiotic compound, designated agrocin 434, has been identified produced by the biocontrol strains K84 and K1026 and a derivative strain, K434, which lacks the ability to produce agrocin 84 (Donner *et al.*, 1993). In addition, Donner *et al.*

(1993) have shown that essential steps in the biosynthesis of agrocin 434 are encoded on what was previously known as the cryptic plasmid of K84 for which they recommended the designation pAgK434. Preliminary studies have shown that agrocin 434 is effective against biovar 2 pathogens. In order to determine the full range of strains inhibited by agrocin 434, strains of *Agrobacterium* belonging to biovars 1, 2 and 3 and *A. rubi* were tested to determine their sensitivity to this novel agrocin.

Some authors have also shown that the choice of media can influence the *in vitro* inhibition of test strains by agrocin producers. For example, Dhanvantari (1983) has demonstrated the sensitivity to agrocin 84 of several strains of the normally resistant biotype 3 agrobacteria using a modified Stonier's medium. In another study, Van Zyl *et al.* (1986) observed marked differences in the *in vitro* susceptibility of pathogenic strains of *A. tumefaciens* to the two agrocin-producing strains K84 and D286 on three different media. This aspect has also been investigated and the importance of adequate pH control via well buffered media has been confirmed.

MATERIALS AND METHODS

Bacterial Strains and Media

Bacterial strains used in this study are listed in Tables 2.1 and 2.2. Details of media preparation are given in Appendix A.

Strains belonging to biovars 1, 2 and 3 were maintained on yeast mannitol (YM) medium (Ellis and Kerr, 1979) while those belonging to *A. rubi* were maintained on yeast extract agar (YEA) (Rodriguez and Tait, 1983) supplemented with biotin (1 µg/ml), calcium pantothenate (0.2 µg/ml) and nicotinic acid (0.2 µg/ml). Cultures were kept at 4°C. When a strain was required a fresh slope was inoculated and incubated at 28°C for 48 hours.

Table 2.1 Bacterial strains tested for sensitivity to agrocin 434.

Strain[a]	Pathogenic	Other designation(s)[b]/source/origin
Biovar 1		
K1	no	ACP-WU11; AG Lockhead, Canada
K24	yes	Kerr, Australia
K31	yes	Kerr, Australia
K57	no	Kerr, Australia
K64	yes	Kerr, Australia
K120	yes	Kerr, Australia
K136	yes	NCPPB-398
K145	yes	NCPPB-396
K187	yes	A6; G Morel, France
K198	yes	WP Roberts, Australia
K230	yes	C58; R Hamilton, USA
K247	yes	T37 NP4; Kerr, Australia
K301	yes	Ach5; J Schell, Belgium
K355	yes	Kerr, Australia
K549	no	Kerr, Australia
K749	no	C58 (p^-); A Kondorosi via J Tempé
K821	yes	Kerr, Australia
K930	yes	A358; E Nester, USA
Biovar 2		
K27	yes	Kerr, Australia
K29	yes	Kerr, Australia
K46	yes	ICPB-TR101; J DeLey
K47	no	ICPB-TR107; J DeLey
K71	yes	Kerr, Australia
K84	no	NCPPB-2407; Kerr, Australia
K107	yes	Kerr, Australia
K108	yes	Kerr, Australia
K114	yes	Kerr, Australia
K118	yes	Kerr, Australia
K123	no	Kerr, Australia
K128	no	Kerr, Australia
K239	yes	Ag43; Panagopoulos, Greece

Table 2.1 Continued.

Strain[a]	Pathogenic	Other designation(s)[b]/source/origin
Biovar 2		
K250	?	Ag40; Panagopoulos, Greece
K352	no	Kerr, Australia
K434	no	Kerr, Australia
K440	yes	California, USA
K441	yes	California, USA
K483	yes	A4; L Moore, USA
K563	yes	ICPB-TR7; France
K566	yes	ICPB-TR101; France
K568	yes	NCIB-8196; Lippincott, USA
K596	yes	ATCC-15834; J Ellis
K602*	yes	ATCC-11325
K640	yes	Kerr, Australia
K744	yes	Kerr, Australia
K745	yes	Kerr, Australia
K746	yes	Kerr, Australia
K970	yes	K Ophel, Australia
K1143	no	D Jones, Australia
Biovar 3		
K252	yes	Ag57; Panagopoulos, Greece
K309*	yes	Kerr, Australia
K374	yes	Kerr, Australia
K375	yes	Kerr, Australia
K376	yes	Kerr, Australia
K377	yes	Kerr, Australia
K521	yes	Kerr, Australia
K995	yes	K Ophel, Australia
K1070	yes	Kerr, Australia
K1072	yes	Kerr, Australia
K1270	no	Burr, USA

Table 2.1 Continued.

Strain[a]	Pathogenic	Other designation(s)[b]/source/origin
A. rubi		
K864	yes	Kerr, Australia
K867	yes	Kerr, Australia
K868	yes	Kerr, Australia
K869	yes	Kerr, Australia
K872	yes	Kerr, Australia
K1046*	yes	ATCC-13335, TR3
K1047	yes	NCPPB1854

[a] Catalogue number in A. Kerr's culture collection.

[b] ATCC, American Type Culture Collection; ICPB, International Collection of Phytopathogenic Bacteria; NCIB, National Collection of Industrial Bacteria (Scotland); NCPPB, National Collection of Plant Pathogenic Bacteria.

* ATCC-11325 (K602), ATCC-13335 (TR3, K1046) and K309 are type cultures of *A. rhizogenes*, *A. rubi* and *A. vitis*, respectively (Ophel and Kerr, 1990).

Table 2.2 Strain K84 and derivatives used in this study.

Strain	Plasmids	Description/origin
K84	pAgK84*	Biological control strain; produces both agrocins
	pAtK84b**	84 and 434.
	pAgK434§	
K434	pAtK84b	Produces agrocin 434.
	pAgK434	
K1143	pAgK434	Produces agrocin 434; D Jones
K1347	–	NC McClure
K1351	pAtK84b	AR Ahmadi
K1352	pAgK84	Produces agrocin 84; AR Ahmadi
K1353	pAgK84	Produces agrocin 84; AR Ahmadi
	pAtK84b	
K1355	pAgK84	Produces both agrocins 84 and 434; AR Ahmadi
	pAgK434	

* Agrocin 84 plasmid (Slota and Farrand, 1982).

** Nopaline catabolic plasmid (Clare *et al.*, 1990).

§ Agrocin 434 plasmid (Donner *et al.*, 1993).

Screening for Sensitivity to Agrocin 434

Agrobacterium strains belonging to biovars 1, 2 and 3 and *A. rubi* were tested for sensitivity to agrocin 434 on three defined media, namely: (1) Stonier's medium (1956) modified by increasing the concentration of phosphates to enhance its buffering capacity; (2) Stonier's medium as modified by Dhanvantari (1983) and (3) mannitol-glutamate (MG) medium (Keane *et al.*, 1970). Descriptions of these media are given in Appendix A. Stonier's medium modified in its phosphate content will be referred to as modified Stonier's medium and Dhanvantari's modification as Dhanvantari's medium. In some of the tests conducted, the pH indicator bromothymol blue (BDH Chemicals), with a pH range of 6.0-7.6, was added to the media at a concentration of 3 mg/l in order to observe qualitative changes in the pH of the medium.

The bioassay technique described by Kerr and Htay (1974) was followed. Agrocin 434 producer strain, K1143, was spot-inoculated on the medium being tested and incubated at 28°C for 48 hours. The producer strain was killed by inverting the plate for 10 min over a filter paper disc saturated with chloroform. The plate was aired for at least 30 min in a laminar flow cabinet to remove residual chloroform. About 4 ml of buffered agar (Appendix A) cooled to about 50°C was then seeded with 1 ml suspension of the test strain and spread over the plate to form a continuous lawn. Plates were incubated at 28°C for 48 hours before being examined for zones of inhibition.

Tests were also conducted in which filter paper discs moistened with aqueous solutions of agrocin 434 partially purified by charcoal adsorption and/or high voltage paper electrophoresis (HVPE) as described below were used in place of agrocin 434 producer colonies.

Isolation and Purification of Agrocin 434

Bacterial cultures were grown for 3-4 days in 10 ml of MMG, a glucose-glutamate liquid medium (Murphy and Roberts, 1979) modified to contain 1.74 g K_2HPO_4 and 5.44 g KH_2PO_4 per liter. The cultures were incubated with shaking at

28°C. Cells were removed by centrifugation and 1.5 ml aliquots of the supernatant were transferred to fresh tubes. To each aliquot was added 100 µl of an aqueous suspension of activated charcoal (15 mg/ml). The mixture was left to stand for 10 min after mixing and then centrifuged for 30 sec to pellet the charcoal. The supernatant was removed by aspiration. The sample was desalted by two successive washes with 1 ml volumes of distilled water. To recover the agrocin, the charcoal was resuspended in 200 µl 70% ethanol, centrifuged and the supernatant collected in a fresh tube. This elution step was repeated twice and the eluants combined and dried under vacuum (SpeedVac SVC 100, Selby Scientific Instruments). The charcoal 70% ethanol desorbates were each dissolved in 10 µl of deionized water for use in bioassays or for further purification.

Agrocin 434 was purified by high voltage paper electrophoresis (HVPE) using the apparatus of Tate (1968). Samples as well as electrophoretic standards (Appendix B and Chapter 5) were applied at 5-10 µl/cm to the center of a 15 cm x 57 cm strip of chromatography paper (Whatman Chr 1). The paper was immersed in the appropriate buffer before loading in the HVPE apparatus. Separations were carried out at 1.8-3 kV for 20-30 min depending on the buffer system used (see Chapter 5 for a more detailed description). Electrophoretograms were dried using a cool air blower and agrocin 434 was detected by absorption of UV light (254 nm Transilluminator UVP).

UV-absorbing bands corresponding to agrocin 434 were cut from the electrophoretograms and placed in a 0.5 ml tube with a pin hole in the bottom. The small tube was then placed in a 1.5 ml tube without a lid, the paper saturated with deionized water and centrifuged. Three successive 50-µl washes resulted in the elution of most of the agrocin. The aqueous solution of agrocin 434 was either concentrated or dried under vacuum and stored at -20°C until needed.

Electrophoretically homogeneous agrocin 434 samples were checked for biological activity using K27 as the indicator strain.

The procedure described was used not only to produce agrocin 434 from strain K1143 for use in bioassays or chemical studies but also for testing other *Agrobacterium* strains for agrocin 434 production.

Plasmid Isolation and Electrophoresis

Agrobacterium plasmids were isolated by a miniprep procedure based on Farrand *et al.* (1985). Bacteria were grown in 5 ml nutrient broth (NB) to late log phase. Cultures were incubated with shaking at 28°C. Cells were harvested from 1.5 ml samples by centrifugation. The bacterial pellet was washed with 1 ml LTE buffer (10 mM Tris-Cl, 1 mM Na_2EDTA, pH 8.0), 100 µl 5M NaCl and 10 µl 10% Na Sarkosyl and centrifuged. After removing the supernatant by aspiration, the pellet was resuspended in 100µl of ice-cold Solution I (50mM glucose, 25 mM Tris-Cl, 10 mM Na_2EDTA, pH 8.0) containing lysozyme (2 mg/ml) and was left on ice for 5 min. Bacteria were lysed by adding 200 µl of Solution II (0.2 M NaOH, 1% SDS) and the contents of the tube mixed by gentle inversion. The lysate was left to stand for 15 min at room temperature and then neutralized by adding 50 µl 2 M Tris-Cl, pH 7.0. It was left to stand at room temperature for another 30 min after which 50 µl 5 M NaCl was added. A single phenol extraction was carried out by adding an equal volume of phenol (saturated with a 3% aqueous solution of NaCl) and maintaining an emulsion for 5 min by gentle inversion. After centrifugation for 10 min, the upper aqueous phase was transferred to a fresh tube with a wide-tipped pipette. DNA was precipitated by adding 0.1 vol 3 M Na acetate, pH 5.2, and 2 vol 100% ethanol. The mixture was left to stand for 15 min at room temperature. DNA was recovered by centrifugation for 10 min also at room temperature. The resulting pellet was washed once with 70% ethanol and dried under vacuum for 5-10 min. It was dissolved in 20 µl LTE buffer containing DNase-free RNase (20 µg/ml) and stored at -20°C until needed.

Plasmids were separated in agarose gels using standard methods (Sambrook *et al.*, 1989), stained with ethidium bromide (5 µg/ml), destained, examined by UV light and photographed using a Polaroid MP4 land camera.

RESULTS AND DISCUSSION

2.1 Screening Agrobacterium Strains for Sensitivity to Agrocin 434

A total of 66 *Agrobacterium* strains (Table 2.1), mostly pathogens, were tested for sensitivity to agrocin 434 on modified Stonier's medium. The results of the screening are summarized in Table 2.3.

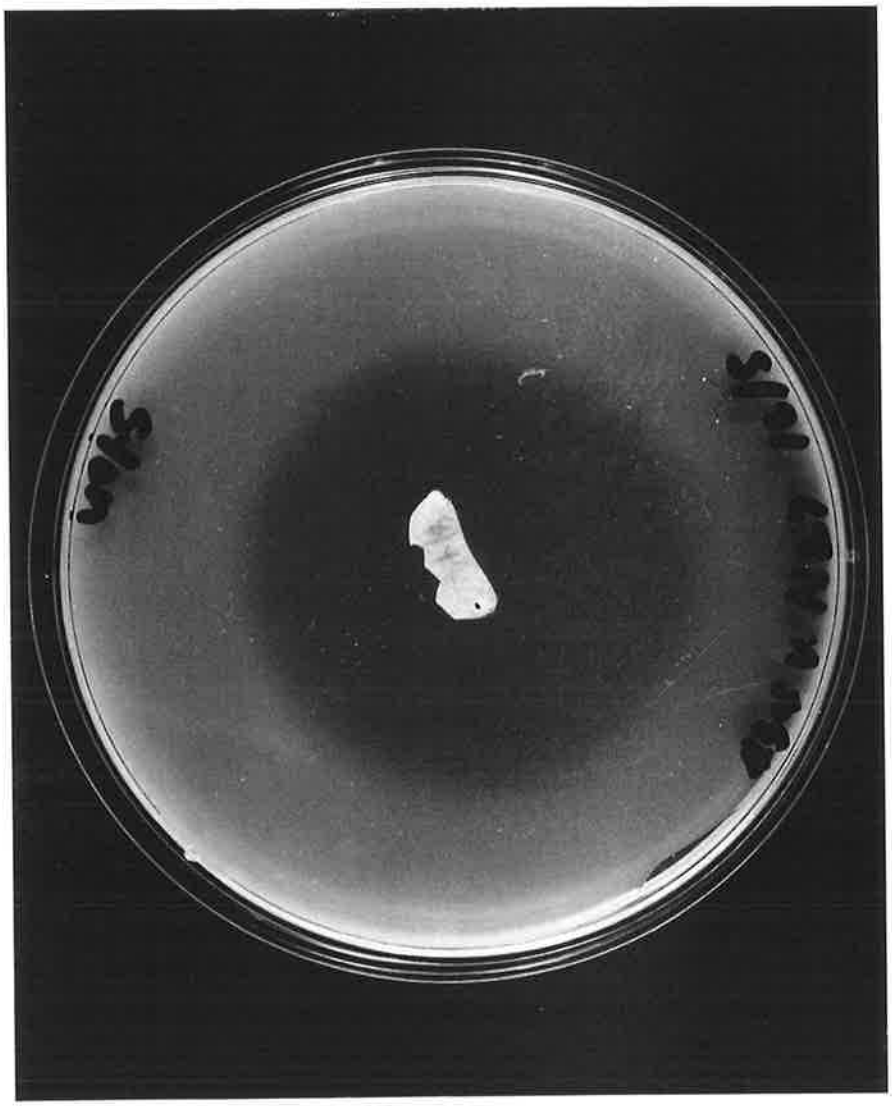
Table 2.3 *In vitro* sensitivity of Agrobacterium strains to agrocin 434.

Strain	Total no. tested	Sensitive	Resistant
Biovar 1	18	0	18
Biovar 2	30	24	6
Biovar 3	11	0	11
A. rubi	7	0	7

In the tests conducted, results obtained with the charcoal desorbates and electrophoretically homogeneous agrocin 434 correlated with those using killed producer colonies. Figure 2.1 shows an agrocin 434 bioassay plate.

All strains of biovars 1 and 3 and *A. rubi* tested were resistant to agrocin 434. In contrast, all biovar 2 strains tested were sensitive with the exception of 6 strains.

Figure 2.1 An agrocin 434 bioassay plate with biovar 2 pathogen K27 as indicator strain. Paper strip contains electrophoretically pure agrocin 434.



Three of these are K84 and its derivatives K434 and K1143, all agrocin 434 producers. The other 3, strains K744, 745 and 746, are Ti-type pathogenic isolates from Australia which were subsequently shown to be agrocin 434 producers themselves. Figure 2.2 shows that the products obtained after isolating agrocins from the pathogenic isolates have the same UV absorption characteristics and electrophoretic mobilities as agrocin 434. Bioassays (Figure 2.3) also showed the same inhibitory characteristics as agrocin 434. In addition each strain was found to contain a plasmid of approximately the same size as pAgK434 as well as a pTi-sized plasmid. As all 3 isolates came from the same source, it is possible that they are identical isolates of one strain.

Sensitivity to agrocin 434 thus appears to be a general characteristic of biovar 2 agrobacteria. The implication is that sensitivity to agrocin 434 may, at least in part, be chromosomally encoded given that the biovar 2 strains tested included nopaline, octopine and agropine strains. In this regard, it may be similar to agrocin J73 (Webster *et al.*, 1986), an agrocin produced by a biovar 2 nopaline strain, J73, active against a broad range of agrobacteria, including grapevine isolates. Webster *et al.* (1986) concluded that sensitivity to agrocin J73 was chromosomally encoded as the agrocin was active even against strains cured of their Ti plasmids. In contrast, sensitivity to agrocin 84 is conferred on the pathogens by their possession of agrocinopine catabolic genes borne on their Ti plasmids (Ellis and Murphy, 1981; Hayman and Farrand, 1988). A broad-host- range agrocin produced by isolate D286 from South Africa, D286 was found to be active against nopaline-, octopine- and agropine-type Ti plasmids (Hendson *et al.*, 1983).

It can be inferred from the resistance of the agrocin 434 producers to the effect of the antibiotic that immunity functions are also encoded on pAgK434. This would be similar to pAgK84 which encodes both agrocin 84 synthetic and immunity functions (Ryder *et al.*, 1987). When pAgK434 was transferred to strain K27, a biovar 2 pathogen sensitive to agrocin 434, the resulting strain was resistant to agrocin 434 and produced agrocin 434 thereby suggesting that immunity is plasmid-encoded (NC McClure, unpublished). However, a plasmidless derivative of K84, strain K1347,

Figure 2.2 Isolated agrocins from pathogenic biovar 2 strains K744 (1), K745 (2) and K746 (3) have UV absorption characteristics and electrophoretic mobilities similar to those of agrocin 434 (indicated by the arrow) produced by K1143 (remaining lanes). M indicates the UV-absorbing marker 2-deoxyadenosine.

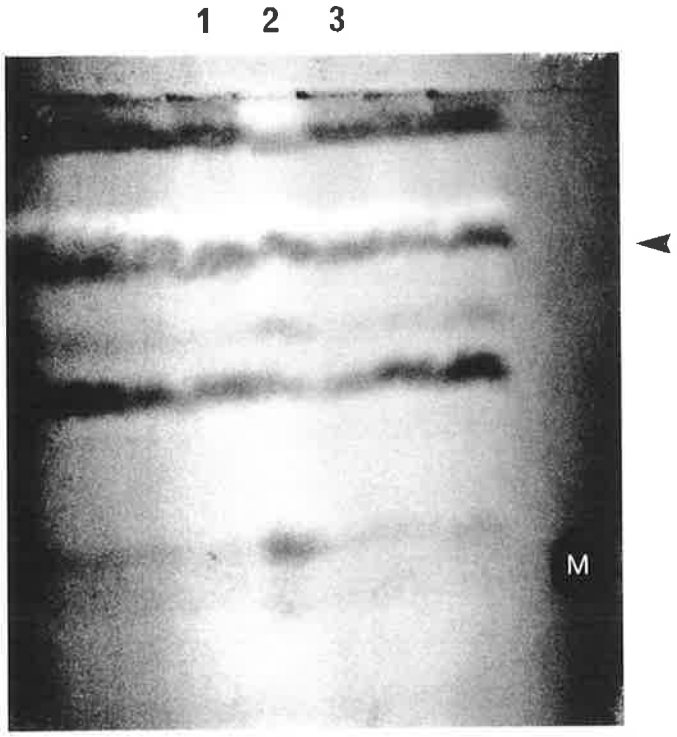
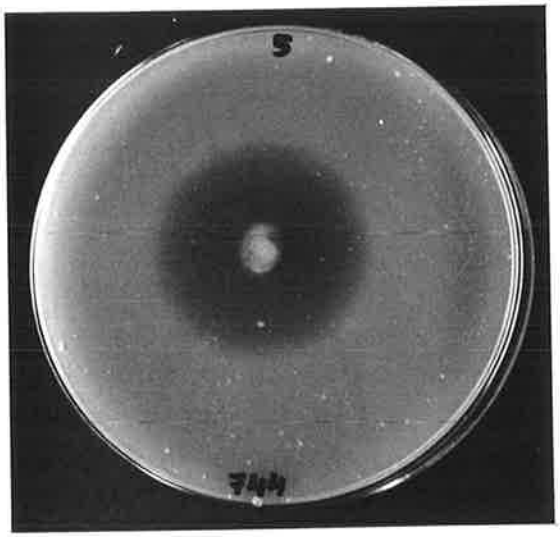
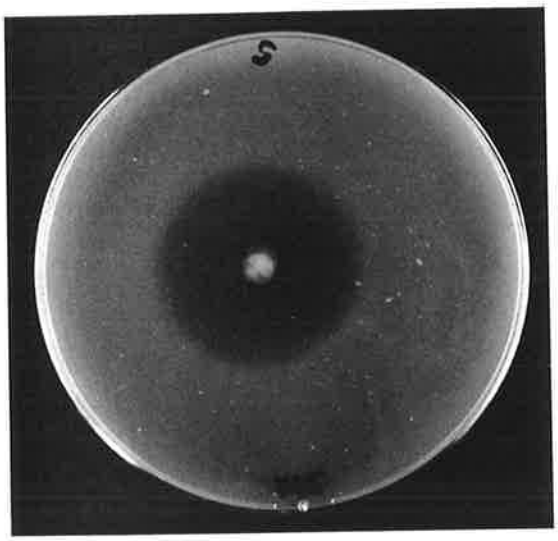

1 2 3
M

Figure 2.3 Inhibitory characteristics of pathogenic isolates K744, K745 and K746, all agrocin 434 producers, tested against biovar 2 pathogen K27.

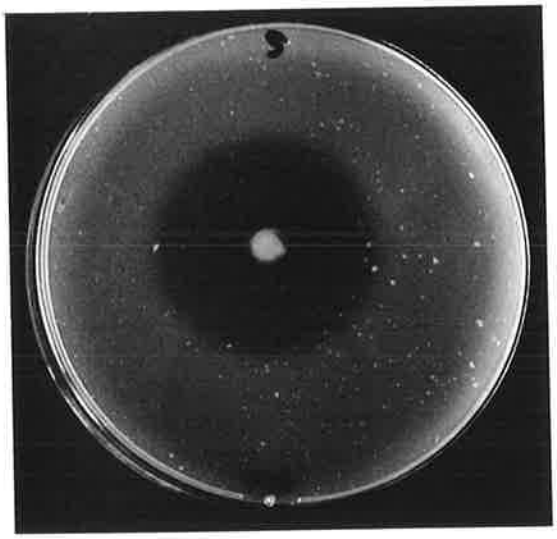
K744



K745



K746



was subsequently shown to be resistant to agrocin 434 suggesting that immunity may also reside on the chromosome.

In order to check if the resistance of non biovar 2 strains to agrocin 434 could be due to the production of this agrocin, representative strains from biovars 1 and 3 and *A. rubi* were tested for agrocin 434 production. None of the strains tested produced agrocin 434 (Table 2.4). Thus, immunity of non biovar 2 strains to agrocin 434 appears to be functionally different from that of the agrocin 434 producers.

Table 2.4 Production of agrocin 434 by non biovar 2 strains.

	Strain	**Agrocin 434 production***
Biovar 1	K120, K136(NCPPB-398)	–
	K230(C58), K301(Ach5)	–
Biovar 3	K252(Ag 57), K309, K374, K377	–
A. rubi	K864, K867, K868, K869, K872,	–
	K1046(ATCC-13335)	–

* +, produces agrocin 434; –, does not produce agrocin 434.

Inhibition zones produced by agrocin 434 were usually smaller in diameter and diffuse when compared to the large and sharp zones obtained with agrocin 84 (Kerr and Htay, 1974) especially when killed producer colonies were used in bioassays. When filter paper discs wet with charcoal desorbates or electrophoretically homogeneous agrocin 434 were used, the zones of inhibition were more clearly discernible.

During the course of the screening experiments, it was noted that strain K27 gave consistent results in bioassays. Thereafter, this strain was used as the indicator strain for all experiments in which the biological activity of agrocin 434 had to be tested.

2.2 Effect of Media on Sensitivity to Agrocin 434

To determine the effect of media on sensitivity to agrocin 434, a subset of strains representing biovars 1, 2 and 3 and *A. rubi* were tested on two other defined media namely, Dhanvantari's medium (Dhanvantari, 1983) and MG (Keane *et al.*, 1970). The results are given in Table 2.5 and include data obtained using modified Stonier's medium for comparison.

In Table 2.5 the response indicated is to both the agrocin 434 producer, K1143, and to electrophoretically homogeneous agrocin 434. For any particular strain, any difference in response to these two is duly noted. Table 2.5 shows no difference in response to agrocin 434 on modified Stonier's and Dhanvantari's media with the exception of the biovar 3 strain K252. This strain showed a zone of inhibition when tested against an agrocin 434 producer colony in the bioassay and none against electrophoretically homogeneous agrocin 434. This suggests that K252 is resistant to agrocin 434 but is sensitive to some other inhibitor(s) secreted by the agrocin 434 producer strain. This will be discussed in Section 2.3.

Modified Stonier's and Dhanvantari's media differ in the following: (1) ammonium nitrate as nitrogen source in Stonier's was replaced by L-glutamic acid in Dhanvantari's; (2) modified Stonier's is more heavily buffered, and (3) Dhanvantari's was supplemented with calcium pantothenate and nicotinic acid. From Table 2.5 it is apparent that these differences did not have any observable effect on the sensitivity of the tested strains to agrocin 434 when tested on the two media.

Table 2.5 Influence of media on the *in vitro* sensitivity of *Agrobacterium* strains to agrocin 434.

Strain		Agrocin 434 response[a,b]		
		Stonier's	Dhanvantari's	MG
Biovar 1	K1	–	–	–
	K24	–	–	–
	K57	–	–	–
	K120	–	–	–
	K136	–	–	–
	K198	–	–	–
	K301	–	–	–
Biovar 2	K27	+	+	–
	K47	+	+	–
	K566	+	+	–
	K602	+	+	–
	K744	–	–	–
	K745	–	–	–
	K746	–	–	–
	K1143	–	–	–
Biovar 3	K252	–	+/–[c]	–
	K377	–	–	–
	K521	–	–	–
	K1070	–	–	–
	K1270	–	–	–
A rubi	K864	–	–	–
	K868	–	–	–
	K869	–	–	–
	K872	–	–	–
	K1046	–	–	–

[a] +, sensitive to agrocin 434; –, resistant to agrocin 434 .

[b] Response is to both the agrocin 434 producer, K1143, and electrophoretically homogeneous agrocin 434, unless otherwise stated.

[c] Sensitive to K1143; resistant to electrophoretically homogeneous agrocin 434.

MG medium differs from modified Stonier's and Dhanvantari's media by the use of mannitol as a carbon source. In addition, it lacks some of the inorganic salts present in the two alternative test media (Appendix A). All the strains tested were found to be resistant to agrocin 434 on MG.

If bioassays were conducted using only agrocin 434 producer colonies, repression of agrocin production could be given as a possible reason for the observed resistance on MG. This is unlikely in this particular case because the same resistance was observed when strains were tested against electrophoretically homogeneous agrocin 434.

The observed resistance to agrocin 434 on MG may be due to inactivation of the agrocin in this medium for as yet unknown reasons. Alternatively, metabolic pathways may be operating in MG medium which give rise to products or changes in cell composition that could account for the resistance observed. Moore (1979) suggested that changes in cell permeability such as those resulting from alteration of the outer membrane protein could influence agrocin sensitivity. In screening experiments conducted by Spiers (1980) in which he also investigated the influence of media on sensitivity to bacteriocin 84, he observed that while the bacteriocin was produced in all the media that he used, for certain media expression of bacteriocin sensitivity was obtained only when agar plugs containing the bacteriocin were inserted into another medium for the sensitivity tests. Again, the implication is that there are components in certain media that inhibit the expression of sensitivity.

Whether the observed resistance on MG is due to inactivation of agrocin 434 or to changes in the test strains induced by the medium is not clear at this point and warrants further investigation. Furthermore, the use of electrophoretically pure agrocin 434 has eliminated the possibility that the observed resistance on MG is due to repression of agrocin production.

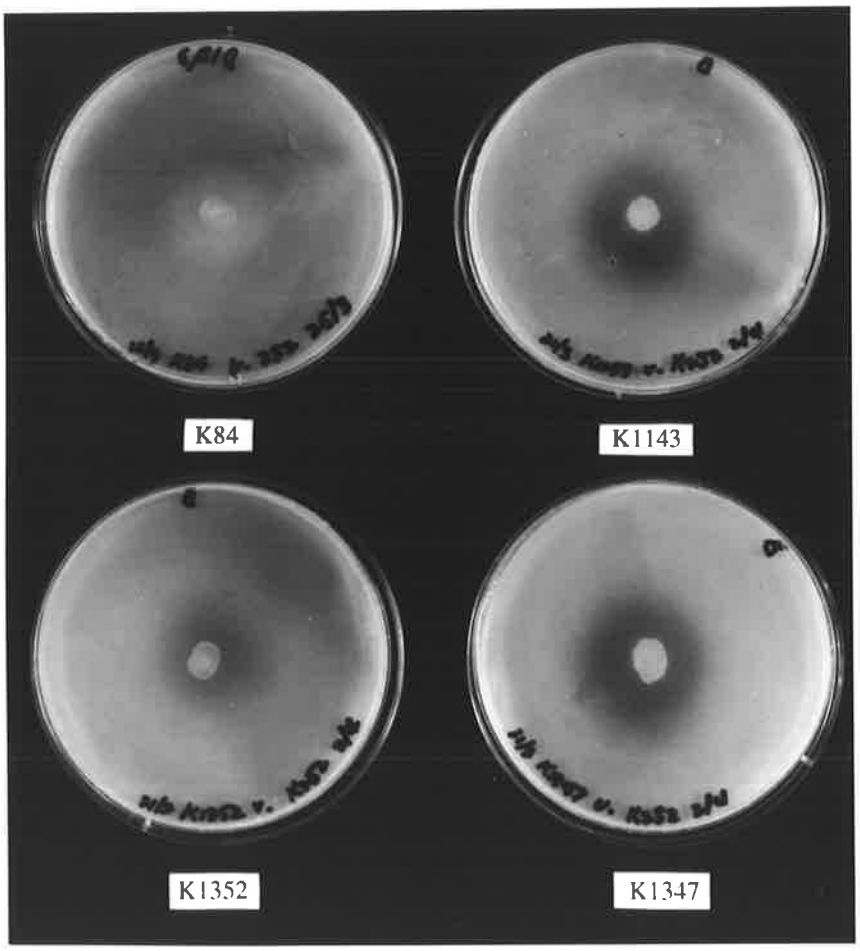
2.3 Effect of Media and pH on Agrocin Bioassays

Prior to testing the influence of media on sensitivity of agrobacteria to agrocin 434, five biovar 3 strains were tested on Dhanvantari's medium for sensitivity to agrocin 84 (Table 2.6). Biovar 3 strains have been shown to be resistant to agrocin 84 *in vitro* and not subject to biological control by K84 (Kerr and Panagopoulos, 1977; Burr and Katz, 1983). However, Dhanvantari (1983) found that several biovar 3 strains were sensitive to agrocin 84 when tested on his own modification of Stonier's basal medium.

The results obtained from the tests, though conducted on only a few strains, support Dhanvantari's findings. Further tests were carried out using strain K252 for the following reasons: (1) it showed a complex inhibition response (Table 2.6); (2) it is an octopine strain and therefore not subject to control by agrocin 84, and (3) it was inhibited by K1143, an agrocin 434-producing strain, when tested on Dhanvatari's medium. In order to find out what could be responsible for the reactions obtained with K252, it was tested against derivatives of K84 which produce either agrocin 84, agrocin 434, both or neither agrocin, on both modified Stonier's and Dhanvantari's media. In addition, since a major difference between the two media is the high buffering capacity of modified Stonier's, a parallel experiment was run in which the two media were supplemented with bromothymol blue, a pH indicator with a range of 6.0-7.6 (yellow-blue) in order to observe qualitative changes in the pH of the media. The results of these experiments are given in Table 2.7 and Figure 2.4.

During the experiment, it was noted that two days after growing the producer strains on plates, a faint halo of blue color was visible around the colonies of K84 and all of its derivatives grown on Dhanvantari's medium supplemented with bromothymol blue. This meant that the area around each producer colony on these plates had become more alkaline as this indicator turns blue when the pH is greater than 7.6. From these results, the observed zones of inhibition on Dhanvantari's medium can be postulated as a pH effect due to growth inhibition of K252 in the alkaline conditions around the producer colonies on a poorly buffered medium. Agrocin 84 cannot be implicated because K252

Figure 2.4 Inhibition of biovar 3 strain K252 by K84 (produces agrocins 84 and 434) and derivative strains K1143 (produces agrocin 434 only), K1352 (produces agrocin 84 only) and K1347 (produces neither agrocin).


K84
K1143
K1352
K1347

is an octopine strain and therefore unable to take up the antibiotic. Neither can the effect be due to agrocin 434 as K252 was found to be resistant when tested against electrophoretically homogeneous agrocin 434 (Table 2.5). The zone of inhibition then that was observed when K252 was first tested against K1143 (Table 2.5) was also likely to be due to the inability of the strain to grow in the alkaline environment surrounding the producer colony. In addition, since K252 was also inhibited by strain K1347, a plasmidless K84 derivative producing neither agrocin 84 nor agrocin 434, the effect of pH provides the simplest explanation for the observed inhibition.

Table 2.6 *In vitro* sensitivity of selected *Agrobacterium* biovar 3 strains to agrocin 84 on Dhanvantari's medium.

Strain	Opine(s)utilized[a]	Observations
K252	octopine	Complex response – concentric zones of growth and inhibition.
K377	nopaline octopine cucumopine	Complex response similar to that observed with K252.
K521	octopine cucumopine	Not sensitive to agrocin 84.
K1070	–	Sensitive to agrocin 84.
K1270	–	Sensitive to agrocin 84

[a] Given where known.

Table 2.7 *In vitro* inhibition of strain K252 by K84 and derivative strains.

Strain	Response[a,b]	
	Stonier's medium	Dhanvantari's medium
K84	–	CR
K434	–	+
K1143	–	+
K1347	–	+
K1351	–	+
K1352	–	+
K1353	–	CR
K1355	–	+

[a] +, inhibited; –, not inhibited by K84 and derivative strains; CR, complex response (see Table 2.6)
[b] Overall poorer growth on Dhanvantari's compared to that on Stonier's medium.

Other reports describing agrocin sensitivity obtained with certain media can also be evaluated in terms of pH effects. For example, Cooksey and Moore (1980) have observed *in vitro* inhibition by K84 of "insensitive" pathogens when grown on potato dextrose agar (PDA) or on a minimal media (mannitol-glutamate) amended with glucose. They have also noted that the effect of glucose was on the sensitivity of the pathogens rather than on the production of agrocin 84. Bouzar and Jones (1992) found that biovar 2 strains produce more acid from glucose than biovar 1 and 3 agrobacteria and developed a simple test for the presumptive identification of biovar 2 strains using this property. It is possible that the inhibition of "insensitive" pathogens that Cooksey and Moore observed was actually due to the inability of those strains to grow under the acidic conditions that resulted from the growth of K84, a biovar 2 strain, on the media containing glucose. Formica (1990) observed a similar acidification of AB medium (McCardell and Pootjes, 1976), one that also contains glucose, when he used it to grow

strain K84. Finally, the inactivation of agrocin 84 at pH's below 4.0 and above 8.0 (Formica, 1990) should be considered when the involvement of this antibiotic compound is being assessed. Pathways for the acid and alkaline degradation of agrocin 84 have been elucidated (Tate *et al.*, 1979).

CONCLUSIONS

A total of 66 strains of *Agrobacterium* belonging to biovars 1, 2 and 3 and *A. rubi* were tested for sensitivity to agrocin 434, another agrocin produced by the biocontrol strains K84 and K1026 and some of their derivatives. Only the biovar 2 strains that do not produce the agrocin were found to be sensitive to agrocin 434. It appears that sensitivity to this novel agrocin is a general characteristic of biovar 2 strains and may, at least in part, be chromosomally encoded.

It was also shown that media had an effect on the expression of sensitivity to agrocin 434. For the particular media tested, whether this is due to inactivation of the agrocin, or to changes in the test strains brought about by growing on a particular medium remains to be clarified. The use of electrophoretically pure agrocin 434 samples has made possible the distinction between inhibition of production and decreased sensitivity in the evaluation of bioassay results.

The study has also reinforced the importance of using sufficiently buffered media so that pH changes are kept to a minimum when investigating aspects of inhibition not related to pH effects.

CHAPTER THREE

GROWTH OF STRAIN K1143 AND AGROCIN 434 PRODUCTION IN A CHEMICALLY DEFINED MEDIUM

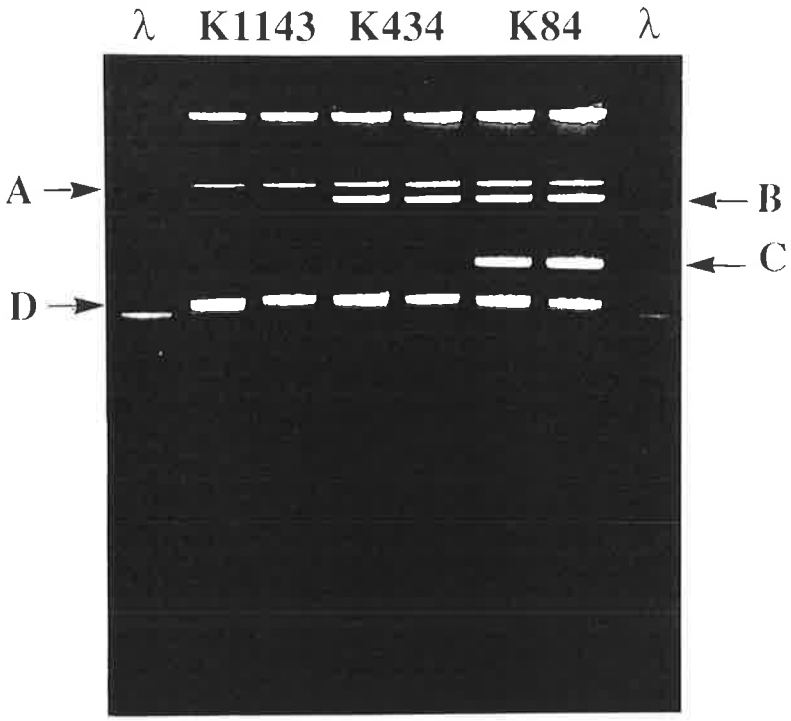
INTRODUCTION

The biocontrol strain K84 carries three plasmids (Figure 3.1): (1) pAgK84, a 48 kb plasmid with genes that encode agrocin 84 synthesis and resistance (Farrand *et al.*, 1985; Ryder *et al.*, 1987; Wang *et al.*, 1994); (2) pAtK84b, a 173 kb plasmid bearing genes for the catabolism of nopaline (Sciaky *et al.*, 1978; Clare *et al.*, 1990); and (3) a large cryptic plasmid (300-400 kb) of previously unknown function (Ellis and Kerr, 1979; Clare *et al.*, 1990). Donner *et al.* (1993) have shown that the cryptic plasmid is involved in the biosynthesis of agrocin 434. They have also isolated a strain, K1143, which carries only this large plasmid now designated pAgK434 (Figure 3.1). For studies on the synthesis of agrocin 434, K1143 is a useful strain to use as it produces only agrocin 434, even though the production of other, as yet unidentified agents cannot be discounted.

The growth of A*grobacterium rhizogenes* strain K1143 in a chemically defined medium and production of agrocin 434 during the growth cycle was investigated. The aim was to obtain information as to how factors such as pH and glucose content of the medium influence the two processes. As the production of agrocin 434 for both biological and chemical studies would have to be scaled up eventually, this information should prove useful when this task is undertaken.

Figure 3.1 Biocontrol strain K84 carries 3 plasmids: (A) pAgK434, previously known as the cryptic plasmid; (B) pAtK84b, the nopaline catabolizing plasmid; and (C) pAgK84, encoding agrocin 84 synthesis and immunity to agrocin 84. Derivative strains K434 and K1143 also carry pAgK434 which encodes functions involved in the biosynthesis of agrocin 434.

D refers to chromosomal DNA; λ to uncut lambda phage DNA.


λ
K1143
K434
K84
λ
A
B
C
D

MATERIALS AND METHODS

Bacterial Strains and Media

Agrocin 434-producing strain K1143 and agrocin 434-sensitive strain K27 have been described in Chapter 2.

Details of bacterial media preparation are given in Appendix A. Bacterial strains were maintained as described in Chapter 2. TY agar (Beringer, 1974) was the preferred medium for the viable counts as colonies tend to aggregate on YM agar. Buffered glucose-glutamate (MMG) was used as the growth and production medium.

Growth and Production Experiments

Inoculum was prepared by inoculating 40 ml of MMG with a loopful of K1143 from a 2-day old culture on YM slope and incubating for 30 hours at 28°C with shaking. 5 ml of this starter culture was used to inoculate 250 ml of MMG in 1-liter culture bottles. The cultures were incubated with shaking at 28°C. These conditions were employed for both the pilot experiment and the main growth and production experiment.

A pilot experiment was conducted (1) to determine the frequency of sampling for the main experiment, and (2) to get an approximate estimate of the dilutions to be used for the viable count. During this pilot experiment, samples were collected at 8-hour intervals up to 96 hours. For each sample, optical density (OD) at 600 nm was measured, glucose detected, and viable count made. Results were then used to plan the main experiment.

During the main experiment, 3-ml samples were collected in 5-ml sterile glass tubes at 2- and 4-hour intervals, depending on the growth rate, up to 56 hours. OD measurements were made of each sample after which aliquots were taken for viable counts and agrocin 434 isolation. The remainder of the 3-ml sample was used for glucose and pH determinations.

Optical density measurements. OD was measured at 600 nm using a Turner Model 330 spectrophotometer. The culture medium MMG was used as the blank.

Viable plate count. The drop count method of Miles and Misra (1938) was used to determine bacterial cell concentrations. At each sampling a 10-fold dilution series consisting of four dilutions (as determined from the pilot experiment) was prepared using sterile buffered saline (Appendix B) as diluent. Three 20-µl drops of each dilution were spotted on TY agar. All four dilutions were plated on one petri dish. The inoculated plates were incubated at 28°C for 24 or more hours until discrete colonies could be counted.

Glucose detection. Glucose was detected by dipping the test area of a glucose strip (Clinistix, Bayer Diagnostics) into the sample and comparing the resulting color with a color chart 10 seconds after wetting. This test gives a semi-quantitative estimation of glucose between the concentrations 14 mM and 28 mM.

pH determination. During the course of the growth experiment, as samples had to be handled aseptically, pH measurements were made using indicator paper (Merck) instead of pH electrodes. At the end of the experiment, however, the pH of the culture was measured using a Metrohm 632 pH meter.

Agrocin 434 production. The production of agrocin 434 was determined on 1.5-ml aliquots from samples collected at 4-hour intervals. The agrocin was isolated and purified using the procedures described in Chapter 2. The UV absorbance of electrophoretically pure samples was measured at 274 nm using a Perkin Elmer Lambda 5 UV/VIS spectrophotometer. Biological activity of the agrocin 434 samples was confirmed by the standard agrocin bioassay (Chapter 2) using Stonier's medium and K27 as indicator strain.

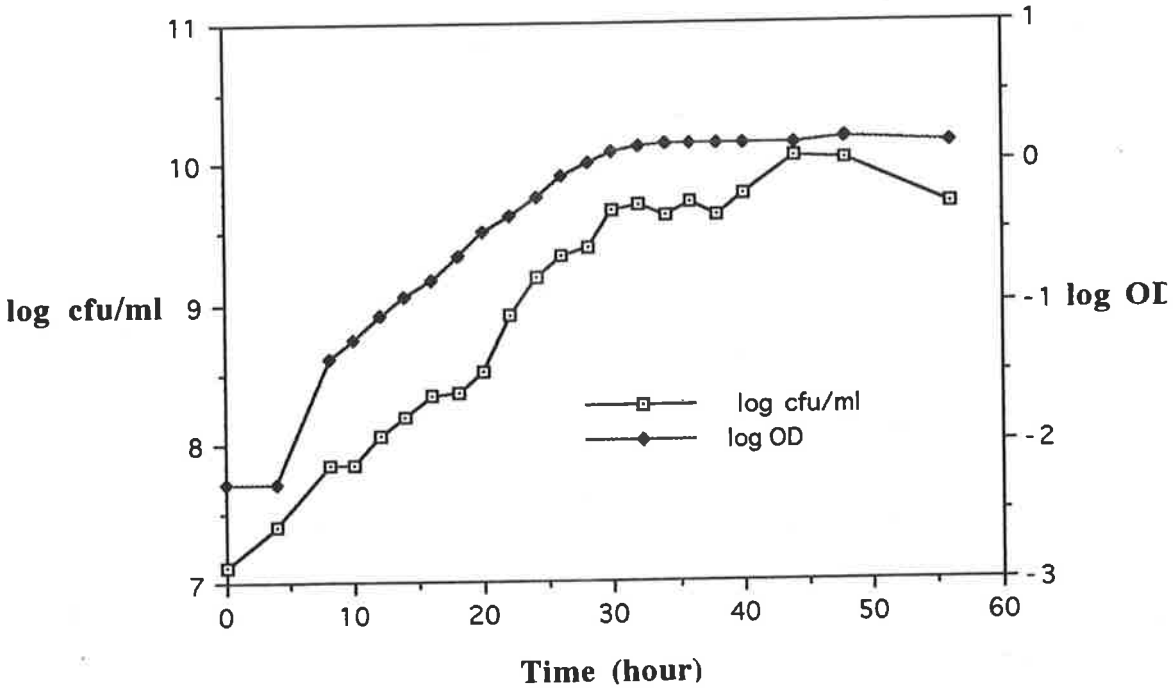
RESULTS AND DISCUSSION

3.1. Growth of Strain K1143 in MMG

Growth curves were derived for K1143 growing in MMG using both OD measurements at 600 nm and viable counts (Figure 3.2). Both curves, one drawn on a linear scale and the other on a log scale, show the expected bacterial growth pattern. Bacterial populations reached maximum values of 10^{10}cells/ml. Viable counts, however, should be treated with caution as they give only the number of cells that are capable of giving rise to a colony on agar under conditions vastly different from those found in the growth medium (Monod, 1949; Wang *et al.*, 1979). For cultures that are well dispersed OD measurement provides a convenient, inexpensive and adequate estimate of bacterial density on a routine basis (Wang *et al.*, 1979).

As a culture grows, metabolic activity of the bacterial cells alters the composition of the original medium. A change in pH is one of the factors that can bring growth to an end (Monod, 1949). The buffering capacity of the medium and the type of metabolites produced by the growing bacteria exert the greatest influence on pH during growth. Bouzar and Jones (1992) have shown that biovar 2 strains of agrobacteria are capable of producing large amounts of acid from glucose and have devised a simple test to demonstrate this characteristic. Since K1143 is a biovar 2 strain and glucose was a component of the growth medium, apppreciable changes in pH could be expected if no measures are taken to correct for this. The pH was measured during the course of the experiment to determine its effect on the buffered medium and consequently on growth and agrocin 434 production. The pH of the cultures decreased from 6.3 at the start of the experiment to 5.8 when it was terminated. The phosphate buffer in the medium had been effective in preventing large changes in pH during the growth of K1143. In an analogous study involving K84, Formica (1990) found a better growth response on a

Figure 3.2 Growth curves derived for K1143 growing in a buffered glucose-glutamate (MMG) medium using OD measurements at 600 nm and viable counts (logarithmic).


log cfu/ml
log OD
11
10
9
8
7
1
0
-1
-2
-3
0
10
20
30
40
50
60
log cfu/ml
log OD
Time (hour)

modified Stonier's medium with phosphate buffer concentrations (50 mM and 100 mM) that resisted changes in pH.

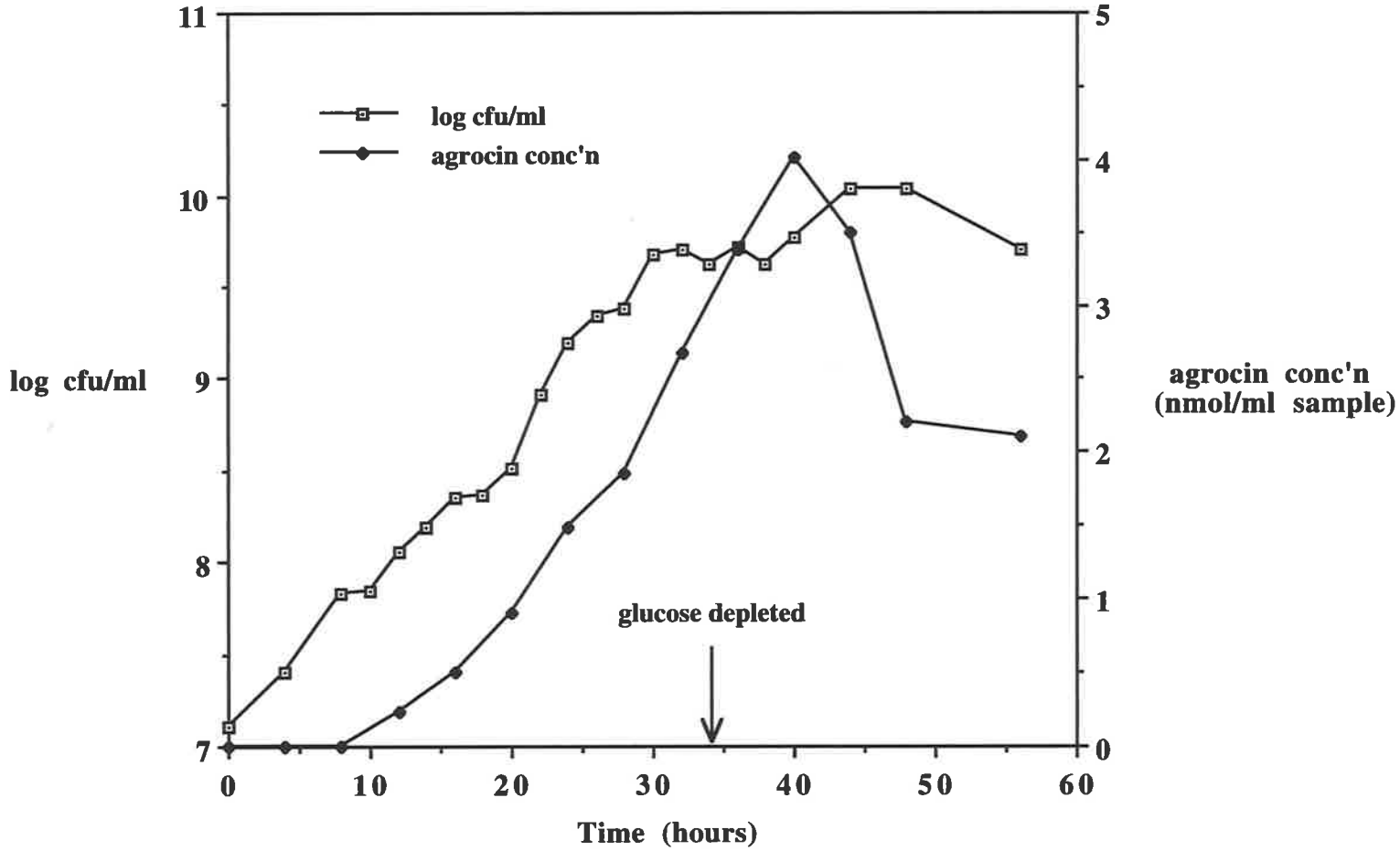
3.2. Agrocin 434 Production in MMG

Agrocin 434 was determined by isolating the compound and measuring its concentration spectrophotometrically instead of the usual method of measuring zones of inhibition produced on bioassay plates. The reason for this is that zones of inhibition produced by agrocin 434 are smaller and much fainter in comparison with the sharp zones obtained with agrocin 84 thus making imprecise the exact correlation of inhibition zone diameter with agrocin 434 concentration.

Figure 3.3 shows that agrocin 434 was produced throughout the growth of K1143. In this respect it is similar to agrocin 84 whose production by strain K84 was shown by Formica (1990) to coincide with the rapid phase of growth. Wang *et al.* (1994) have also demonstrated the expression of all five *agn* (agrocin 84) genes at all growth stages in both Stonier's minimal medium and AB minimal medium. By definition, the antibiotic activity of both agrocins classifies them as secondary metabolites (Vining, 1995; Wang *et al.*, 1979). However, their constitutive production suggests otherwise.

Agrocin 434 production started well before glucose depletion was noted (Figure 3.3) so clearly its production was not triggered by nutrient depletion, which activates many secondary metabolic processes (Vining, 1995). The decline in agrocin production after 40 hours could probably be attributed to the cultures being in the last stages of the growth cycle. It appears that agrocin 434 production is not strongly regulated by glucose. Ryder *et al.* (1987) have reported that glucose had no effect on agrocin 84 production. Similarly, Wang *et al.* (1994) have observed that the expression of the *agn* genes do not appear to be influenced by carbon catabolite repression. In contrast, the biosynthesis of the antibiotic oomycin A, responsible for biocontrol by

Figure 3.3 Agrocin 434 production by strain K1143 in buffered glucose-glutamate (MMG) medium.


log cfu/ml
agrocin conc'n
11
10
9
8
7
5
4
3
2
1
0
log cfu/ml
agrocin conc'n
(nmol/ml sample)
glucose depleted
0
10
20
30
40
50
60
Time (hours)

Pseudomonas fluorescens HV37a of *Pythium ultimum*-induced damping off disease, is regulated by glucose (James and Gutterson, 1986: Gutterson *et al.*, 1988).

In his work on agrocin 84 production by strain K84, Formica (1990) has investigated the effect of factors such as type of carbon source and supplementation with opines, vitamins, cytokinins and acetosyringone on growth and agrocin 84 production. Similarly, Wang *et al.* (1994) have determined the influence of carbon sources, pH and the addition of opines and root exudates to the medium, on the expression of the *agn* genes. Future investigations on the production of agrocin 434 may be conducted along similar lines in order to gain a more detailed understanding of the factors that influence the synthesis of this novel agrocin. Such studies would be aided by a detailed molecular analysis of the agrocin 434 synthesis genes.

CONCLUSIONS

The glucose-glutamate medium MMG supports both the growth of strain K1143 and agrocin 434 production. No large changes in pH were observed in this phosphate buffered medium. Agrocin 434 was produced throughout the growth cycle even though its antibiotic activity is suggestive of a secondary metabolite. In addition, it appears that, like that of agrocin 84, the production of agrocin 434 was not strongly regulated by glucose concentration.

CHAPTER FOUR

RESTRICTION ENDONUCLEASE MAPPING OF PLASMID pAgK1318

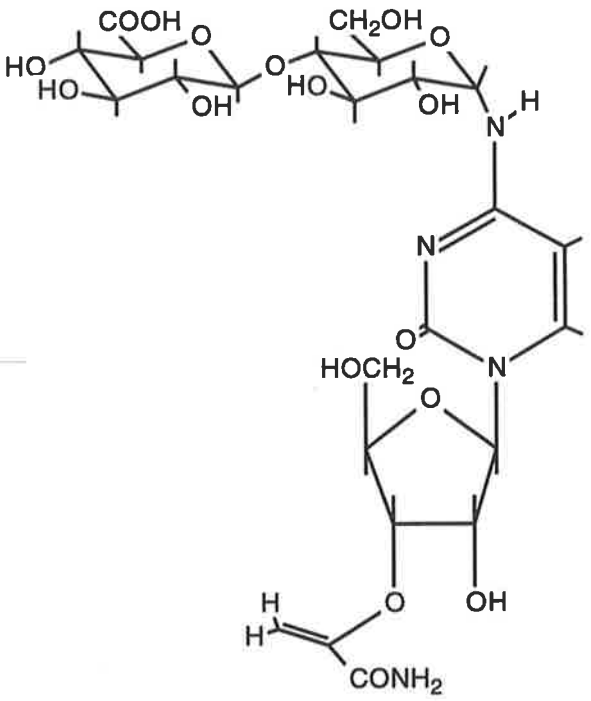
INTRODUCTION

The involvement of pAgK434 in the biosynthesis of agrocin 434 is the first function assigned to what was previously known as the cryptic plasmid carried by the biocontrol strain K84 and some of its derivatives (Donner *et al.*, 1993). The objective of this study was to provide evidence to localize the relevant genes on this large plasmid (300-400 kb). Many approaches can be taken to achieve this objective, e.g. the production of transposon insertion mutants and molecular cloning of functional regions. Genetic characterization can also be facilitated by the availability of a physical map of the whole plasmid or at least relevant regions of it.

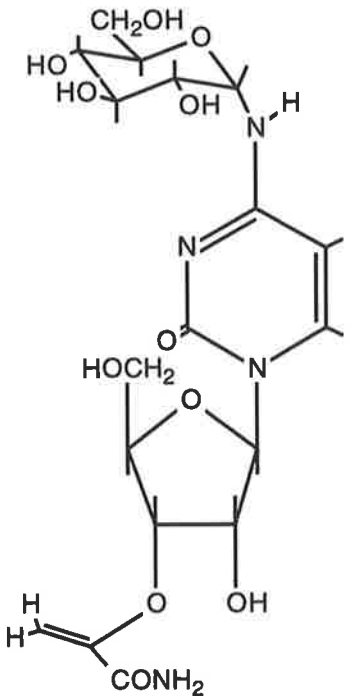
Mapping a plasmid of the size of pAgK434 can be laborious and difficult. Fortunately, a derivative of K84, strain K1318, carrying a deleted version of pAgK434 had been isolated (Jones and Rosewarne, unpublished). Strain K1318 resulted from an attempt to isolate Tn*5* insertion mutants in the agrocin 434 biosynthetic pathway. However, it has subsequently been shown that strain K1318 carries a Tn*5* insertion in the chromosome and that loss of ability to produce agrocin 434 is the result of a single large deletion in plasmid pAgK434 (NC McClure, personal communication).

Strain K1318 produces a biologically inactive fragment of agrocin 434, designated nucleoside 4176, which is missing a uronic acid substituent (Figure 4.1). It has been shown that functions involved in the synthesis of this inactive fragment of agrocin 434 are encoded on the deleted plasmid (designated pAgK1318). If the genes

Figure 4.1 The biologically inactive fragment of agrocin 434 (Donner SC, unpublished), designated nucleoside 4176, lacks the glucuronic acid component attached to glucose via a β-1,4 linkage in agrocin 434.



Proposed AGROCIN 434



NUCLEOSIDE 4176

responsible for the synthesis of agrocin 434 are clustered, as is the case for agrocin 84 (Farrand *et al.*, 1985; Ryder *et al.*, 1987; Wang *et al.*, 1994), genes encoding those functions would most probably be located in the vicinity of the deletion. The smaller size of pAgK1318 as compared to pAgK434 has facilitated the production of a restriction map for this deleted plasmid.

This chapter describes the mapping of a region of pAgK1318 which is likely to contain some of the genes involved in the biosynthesis of agrocin 434 and which will enable construction of a complete restriction map of pAgK1318. The map could prove useful not only in localizing the genes that are involved in the biosynthesis of agrocin 434 but also in studying other, as yet unidentified, functions encoded on pAgK1318 and pAgK434.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

Bacterial strains and plasmids used in this study are listed in Table 4.1. Details of media and antibiotic preparation are described in Appendix A. Strain K1318 was maintained on YM supplemented with kanamycin, Km, at a concentration of 200µg/ml. For plasmid preparations, K1318 was grown in YEB (Van Larebeke *et al.*, 1977), also with Km, at 28°C. Strain JM109 was grown at 37°C in LB broth (Miller, 1972) and maintained on LB agar. Prior to transformation, JM109 was grown on M9 minimal medium (Sambrook *et al.*, 1989). Ampicillin, Amp, was added to give a final concentration of 50 µg/ml where appropriate.

Isolation and Purification of Plasmid DNA

At the start of this study, a large scale alkali lysis method based on the procedures of Casse *et al.* (1979) and Farrand *et al.* (1985) was used for the preparation

of plasmid DNA from K1318. Cultures were grown in NB (Oxoid) medium with Km. Plasmid DNA was then recovered from low melting point agarose gels (Boehringer-Mannheim or SeaPlaque) by treatment with the enzyme agarase (Boehringer-Mannheim) following the manufacturer's instructions. This large scale method proved to be unsatisfactory for K1318, giving very variable and often low yields.

Table 4.1 Bacterial strains and plasmids used in experiments described in this chapter.

Strain	Plasmid	Description
A. rhizogenes K1318	pAgK1318	Cured derivative of K434 (pAgK84$^-$) with Tn*5* insertion in chromosome and deletion in plasmid pAgK434, (DA Jones and G Rosewarne, unpublished).
E. coli JM109	—	Host strain carrying *lac*1^{q}ΔM15 on an F' episome for blue/white color screening, (Yanisch-Perron *et al.*, 1985), gift from JP Rao, also supplied by Stratagene as competent cells (Epicurian Coli®JM109).
—	pUC18	2.7 kb, Amp^r, (Yanisch-Perron *et al.*, 1985), gift from NC McClure.
—	pGEM®-7Zf(+)	3.0 kb, Amp^r, supplied by Promega.

Modifications were then made to the miniprep procedure for *Agrobacterium* plasmids described in Chapter 2 and the resulting method was used for the remainder of

the study. In this method, bacterial cultures were grown for 24 hours in 10 ml YEB. Cells were collected by centrifugation in 10ml tubes at 9000 rpm for 5 min using a Sorvall centrifuge (Superspeed RC2-B). The bacterial pellet was washed with 1 ml LTE buffer (10mM Tris-Cl, 1mM Na_2EDTA, pH 8.0), 100µl 5 M NaCl and 20µl 10% Na Sarkosyl and then centrifuged. The supernatant was removed by aspiration and the pellet was resuspended in 200µl of ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM Na_2EDTA, pH 8.0) containing lysozyme (2mg/ml). The suspension was left on ice for 5min. Bacteria were lysed by adding 400 µl of Solution II (0.2 M NaOH, 1% SDS) and incubated at 34°C for 20 min. The suspension was neutralized by adding 100 µl 2 M Tris-Cl, pH 7.0, mixed and then left to stand at room temperature for 30 min. The lysate was adjusted to about 3% NaCl (w/v) by adding 100µl 5 M NaCl. An equal volume (800 µl) of phenol (saturated with a 3% aqueous solution of NaCl) was added. An emulsion was maintained for 5 min by gentle inversion. Using a wide-tipped pipette, the upper aqueous phase was collected in a fresh tube after centrifugation for 10 min. The DNA was precipitated by adding an equal volume of isopropanol and recovered by centrifuging for 15 min at room temperature. The pellet was washed once with 70% ethanol and dried under vacuum for 5-10 min. It was then dissolved in 20 µl LTE buffer containing DNase-free RNase (20 µg/ml) and stored at -20°C until needed. In this procedure all mixing were done by gentle inversion to avoid shearing the plasmid DNA. Crude plasmid preparations were then run in low melting point agarose gels and plasmid DNA was recovered by treatment with agarase.

To isolate recombinant plasmids from *E. coli*, the miniprep procedure of Sambrook *et al.* (1989) was followed.

Restriction Endonuclease Digestion

Restriction enzymes were obtained from Boehringer-Mannheim. Digestions were carried out according to the manufacturer's instructions. Partial digests were obtained by limiting the reaction time so that not all possible sites in the DNA are

cleaved by the particular enzyme. When double digests were required, the DNA was incubated with the two enzymes of interest in the buffer most suitable for the double digest following the manufacturer's recommendations. DNA fragments, especially large ones (>9 kb), were recovered from low melting point agarose gels by agarase treatment.. In addition, small restriction fragments were purified using a Geneclean® kit (Bio 101, La Jolla, California). When DNA fragments required no separation, the mixture was purified by phenol/chloroform (TE-saturated phenol/chloroform 1:1) extraction followed by ethanol precipitation.

Electrophoresis of DNA

DNA molecules were separated in agarose gels using standard electrophoretic methods (Sambrook *et al.*, 1989). The following were used as molecular size standards: (1) uncut λ phage DNA (Sigma), (2) a mixture of fragments from the cleavage of λ with *Hin*dIII (Promega), and (3) the 1 Kb DNA ladder (Gibco BRL). After electrophoresis, gels were stained with ethidium bromide (5μg/mL), destained, examined by UV light and photographed.

Molecular Cloning

Ligations. DNA ligations were carried out using T4 DNA ligase (Boehringer-Mannheim) in the supplied buffer. Ligation mixtures were incubated overnight at 15°C.

Preparation of competent cells and transformation. *E. coli* JM109 was the host strain used in transformation experiments. Competent cells were prepared following the method described by Promega (Protocols and Applications Guide, 1991, Promega Corp.). The transformation protocol described by Sambrook *et al.* (1989) was followed. When commercially prepared competent cells were used, transformations were carried out according to the manufacturer's instructions. Transformation mixtures

were plated on H-agar (Appendix A) supplemented with Amp, X-Gal and IPTG. Recombinant colonies were white.

Analysis of recombinant plasmids. Recombinant plasmids were analyzed for the correct size of insert DNA by digestion with the appropriate restriction enzyme and separation in agarose gels.

Southern Analysis

Southern blotting. With minor modifications, the capillary transfer method (Southern, 1975) as described by Sambrook *et al.* (1989) was used to transfer DNA to positively-charged nylon membranes (Boehringer-Mannheim). The specific procedure involved transferring DNA from a single gel simultaneously to two nylon membranes. DNA was fixed by baking at 120°C for 30 min.

DNA labelling. DNA probes were prepared by random primed labelling with digoxigenin-11-dUTP (DIG-11-dUTP). The labelling reactions were performed using the DIG DNA Labeling Kit (Boehringer-Mannheim).

Hybridization. Hybridizations were done according to the protocol described in The DIG System User's Guide for Filter Hybridization (Boehringer-Mannheim) with some modifications. To serve as blocking agent, powdered skim milk was used to a final concentration of 2% (w/v) in the hybridization buffer. Hybridization reactions in 50% formamide were carried out overnight at 42°C in a shaking water bath (The Belly Dancer/Hybridization Water Bath, Stovall, Life Science Inc., Greensboro, NC, USA). For washing at high stringency, membranes were washed twice with 2X SSC, 0.1% SDS (w/v) for 5 min at room temperature then twice with 0.1X SSC, 0.1% SDS (w/v) for 15 min at 68°C. Washes were performed with gentle agitation.

Chemiluminescent detection and autoradiography. DIG-labelled DNA was detected by using the chemiluminescent substrate Lumigen™PPD [4-methoxy-4-(3-phosphate-phenyl)-spiro(1,2-dioxetane-3,2'-adamantane)disodium salt] (Boehringer-Mannheim) following the manufacturer's instructions. The procedure involved immuno-

detection of hybridized probes with an alkaline phosphatase-conjugated Anti-digoxigenin antibody.

Membranes were exposed to X-ray film (Kodak X-Omat™) between intensifying screens. Exposures were carried out at room temperature. Exposure times, usually between 5 min to 2 hours, were adjusted to obtain the desired signal intensity.

RESULTS AND DISCUSSION

4.1 Isolation and Purification of Plasmid DNA

In order to facilitate the mapping experiments, an efficient method was needed to isolate and purify plasmid DNA from strain K1318. The large scale procedure (Casse *et al.*, 1979; Farrand *et al.*, 1985) used at the start of the study did not work well for this particular strain. Yields were very low and varied greatly from batch to batch. Some batches yielded no useful products. A method was then developed by scaling up an alkaline lysis procedure based on Farrand *et al.* (1985) routinely used in our laboratory for the minipreparation of *Agrobacterium* plasmids. The procedure worked for pAgK1318 giving 4-5 times greater yields for the same volume of original cultures compared to the large scale method used initially. In addition to being reliable, the method is simple. All operations after centrifugation of the bacterial cells can be performed at the bench.

Purified plasmid DNA was recovered from low melting point agarose gels by digesting agarose with the enzyme agarase. Average recovery is about 70-85% of good quality plasmid DNA that is suitable for cloning and restriction endonuclease analysis. The method was also chosen for its simplicity as compared to purification by isopycnic ultracentrifugation in CsCl-ethidium bromide (EtBr) gradients which is time consuming and requires more expensive instruments and chemicals. Furthermore, agarase is not a

health hazard like EtBr which, though only moderately toxic, is a powerful mutagen (Sambrook *et al.*, 1989).

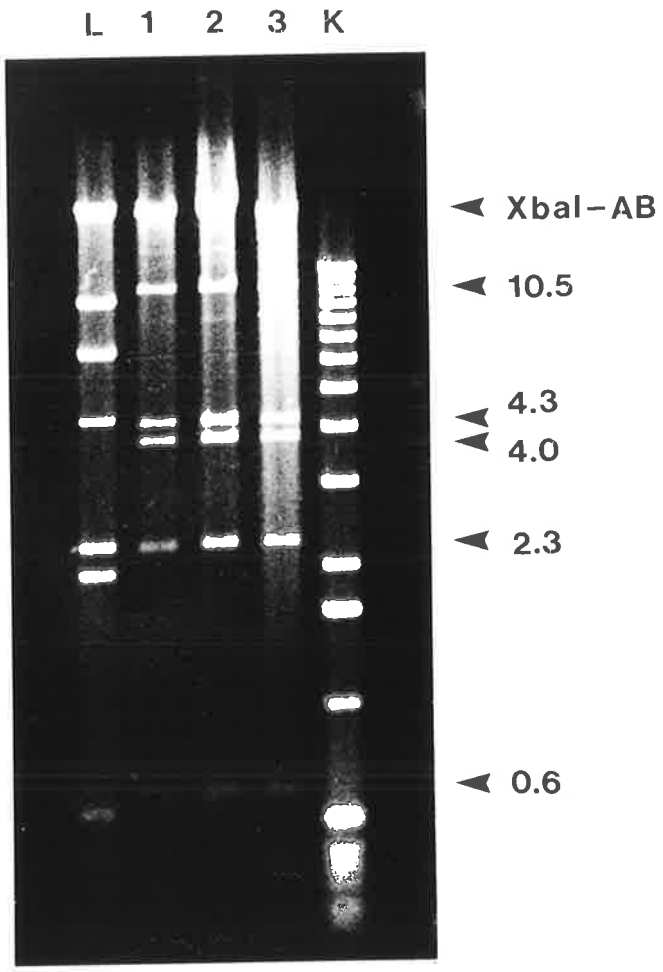
4.2 Genetic Mapping

To select the enzymes with which to map pAgK1318, the plasmid was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Sph*I and *Xba*I. *Xba*I gave the least number of identifiable fragments (7), followed by *Sma*I (10), with the remaining enzymes generating more. *Xba*I was chosen to commence the mapping of pAgK1318. The restriction pattern on cutting pAgK1318 with *Xba*I is shown in Figure 4.2. The largest fragments (designated *Xba*I-AB for convenience) were thought to be either identical or very similar in size as they were not resolved even by extensive electrophoresis on long gels. These fragments were isolated from low melting point agarose gels and further digested with the restriction endonucleases *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Nsi*I, *Pst*I, *Sac*I, *Sal*I and *Sma*I. *Sma*I gave the least number of identifiable fragments (8) with the rest giving 12 or more. For the initial genetic manipulations of *Xba*I-AB, *Hin*dIII was chosen. As cloning was part of the experiments planned, it would be easier to clone *Hin*dIII fragments that generate cohesive ends than *Sma*I fragments that carry blunt ends.

For the mapping experiments, the smaller *Xba*I fragments (≤ 10.5 kb) as well as the fragments generated by *Hin*dIII digestion of *Xba*I-AB were subcloned. From the subcloning of the *Xba*I-AB*Hin*dIII fragments, four distinct clones were obtained in which the insert DNA had a *Hin*dIII end and an *Xba*I end. This supported the assumption that *Xba*I-AB consists of two fragments. DIG-labelled probes of all the *Xba*I fragments were prepared.

With some modifications, the technique of Danna (1980) was used in ordering the *Xba*I fragments through partial digests. In Danna's procedure, individual partial digestion products were purified from large-scale digests and then incubated with excess enzyme to complete the digestion. Identification of the fragments after complete

Figure 4.2 *Xba*I restriction digestion pattern of plasmid pAgK1318 (lanes 1, 2 and 3). Fragments were separated on 0.7% agarose gel. L, λ *Hin*dIII; K, 1 Kb DNA ladder.


L 1 2 3 K
XbaI-AB
10.5
4.3
4.0
2.3
0.6

digestion enabled one to determine which final products were contained in a given partial digest. In this study, partial digests were analyzed by Southern hybridization using the DIG-labelled probes. Figure 4.3, for example, shows hybridization of the partial digest with the *Xba*I 4.0 and 4.3 kb probes. Similar hybridizations were carried out with the remainder of the probes. The composition of a specific partial digestion product was determined by which probes hybridized to it. Using the results given in Table 4.2, the *Xba*I fragments were ordered with the exception of the largest fragments which could not be resolved (Figure 4.4). All the smaller fragments were found to be contiguous.

Table 4.2 Analysis of *Xba*I partial digestion products by hybridization with DIG-labelled *Xba*I fragments.

Size of partial digestion product (kb)	**Probes hybridizing to product (kb)**	**Total (kb)**
21	10.5, 4.3, 4.0, 2.3, 0.6	21.7
18.9	10.5, 4.3, 4.0	18.8
17.5	10.5, 4.0, 2.3, 0.6	17.4
14.5	10.5, 4.0	14.5
13.5	10.5, 2.3, 0.6	13.4
12.8	10.5, 2.3	12.8
8.4	4.3, 4.0	8.3
2.9	2.3, 0.6	2.9

Figure 4.3 *Xba*I partial digest (P) of pAgK1318 and autoradiograms of Southern blots probed with the DIG-labelled 4.0 (2) and 4.3 (1) kb *Xba*I fragments. Similar hybridizations of the partial digest were carried out using the DIG-labelled 10.5, 2.3 and 0.6 kb *Xba*I fragments as probes.

K, 1 Kb DNA ladder; L, λ *Hin*dIII.

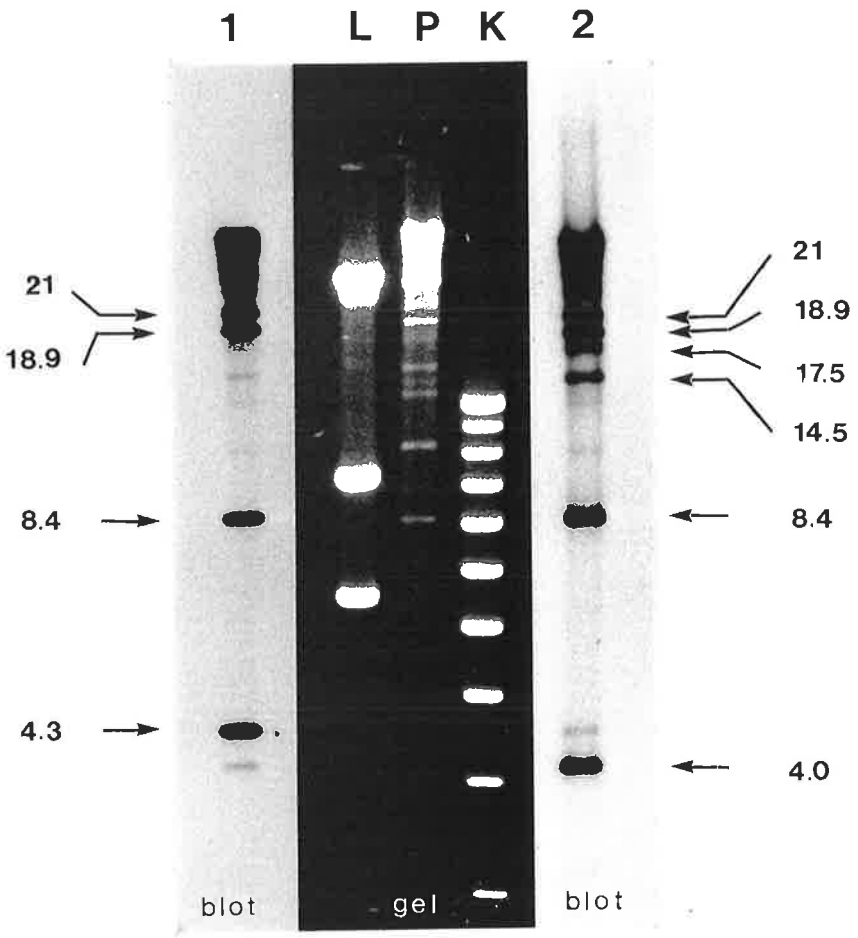
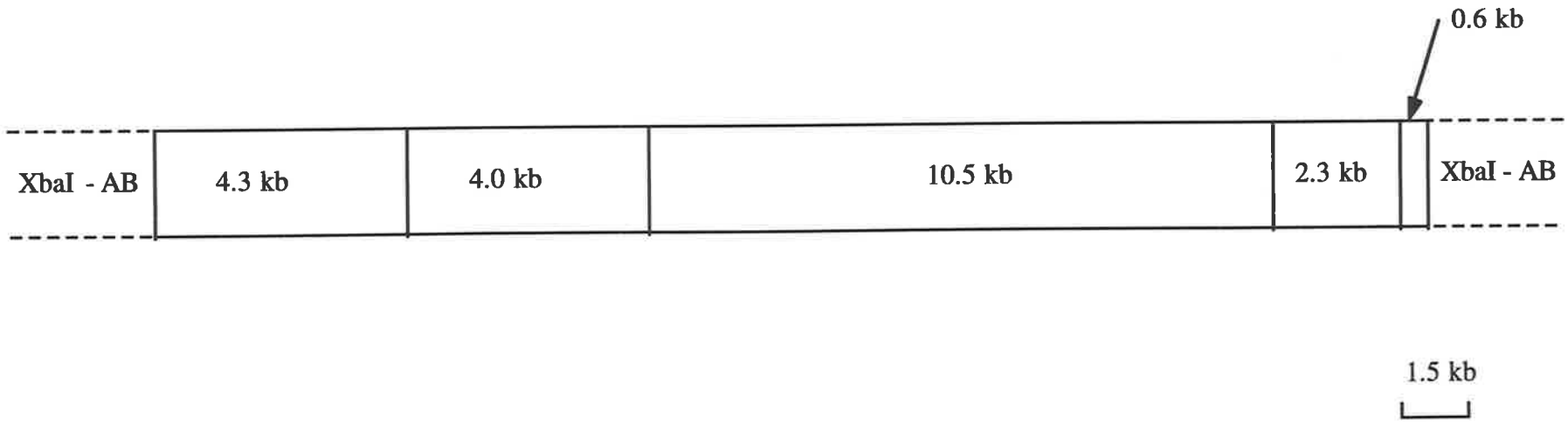

1
L
P
K
2
21
18.9
8.4
4.3
21
18.9
17.5
14.5
8.4
4.0
blot
gel
blot

Figure 4.4 Plasmid pAgK1318 *Xba*I fragments ordered through Southern analysis of partial digestion products.


0.6 kb
XbaI - AB
4.3 kb
4.0 kb
10.5 kb
2.3 kb
XbaI - AB
1.5 kb

When *Xba*I digests of pAgK434 and pAgK1318 were compared, the results supported the assumption that a single large deletion event had given rise to plasmid pAgK1318. A novel 10.5 kb *Xba*I fragment of pAgK1318 encompasses the site of deletion (NC McClure, unpublished) and could be expected to contain at least some of the genes involved in the production of the biologically inactive fragment of agrocin 434. This assumption is dependent on the clustering of genes involved in agrocin 434 production, which will only be confirmed by further investigations. Fine mapping of the region inclusive of, and flanking, the novel *Xba*I fragment was carried out with respect to the restriction endonucleases *Bam*HI, *Hin*dIII and *Sma*I.

Southern analysis of complete *Bam*HI, *Hin*dIII and *Sma*I digests of pAgK1318 were performed using the DIG-labelled probes (Figure 4.5). The results of these hybridizations (Table 4.3) were used to order the relevant fragments of each of the three digests with respect to the *Xba*I fragments. The accuracy of the order obtained for each restriction enzyme was checked by digesting each *Xba*I fragment with that enzyme (Figure 4.6). Additional smaller fragments of the *Hin*dIII digest within the novel 10.5 kb *Xba*I fragment that were missed during Southern analysis were revealed by the *Hin*dIII digestion of the 10.5 kb fragment. Table 4.4 summarizes the results of these multiple enzyme digestions. As a further check on the internal consistency of the map obtained, a Southern blot of the complete *Bam*HI, *Hin*dIII and S*ma*I digests of pAgK1318 was probed with the DIG-labelled *Xba*I-AB. Results are given in Table 4.5 and incorporates information from Southern analysis using the smaller *Xba*I fragments. It shows that *Xba*I-AB hybridized only with those fragments that did not hybridize with the alternative *Xba*I probes except in certain cases where the fragments involved are found at the borders of the region being mapped.

Figure 4.5 Hybridization of complete *Bam*HI (3), *Hin*dIII (2) and S*ma*I (1) digests of pAgK1318 with the DIG-labelled 10.5 kb *Xba*I fragment. Similar hybridizations were carried out using DIG-labelled probes made from the remaining *Xba*I fragments of pAgK1318.

K, 1 Kb DNA ladder.

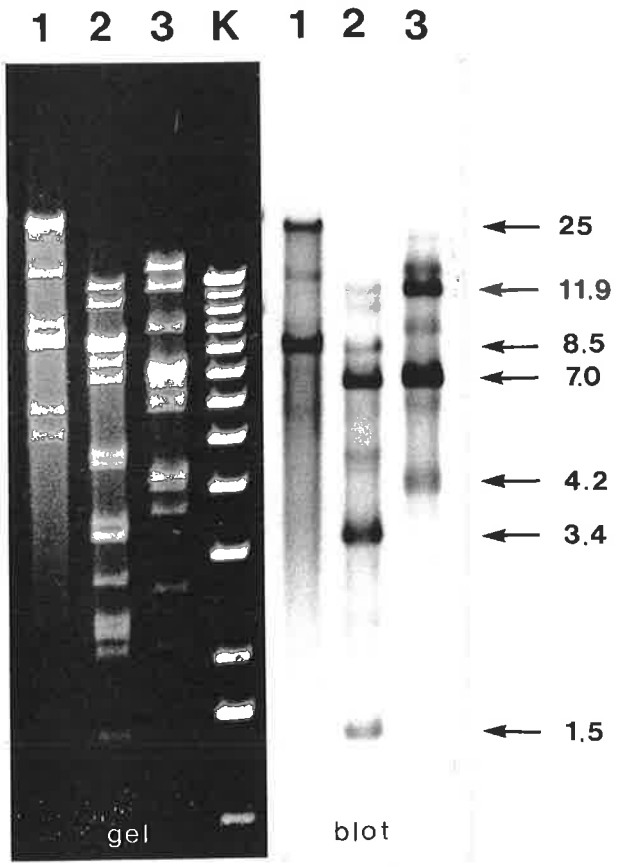
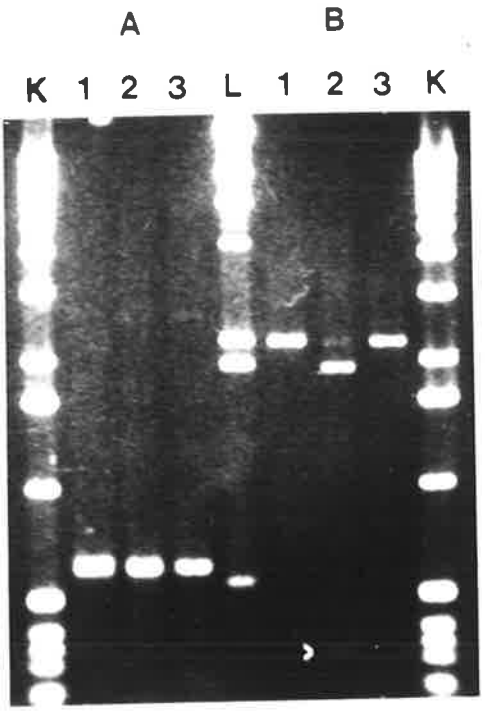

1 2 3 K 1 2 3
25
11.9
8.5
7.0
4.2
3.4
1.5
gel
blot

Figure 4.6 Digestion of pAgK1318 *Xba*I fragments 0.6 kb (A), 2.3 kb (B), 4.0 kb (C) and 4.3 kb (D) with the restriction endonucleases *Bam*HI (3), *Hin*dIII (2) and *Sma*I (1). Fragments were separated on 2% agarose gels. Similar hybridizations were carried out with the 10.5 kb *Xba*I fragment.

K, 1 Kb DNA ladder; L, λ *Hin*dIII.

Gel 1



Gel 2

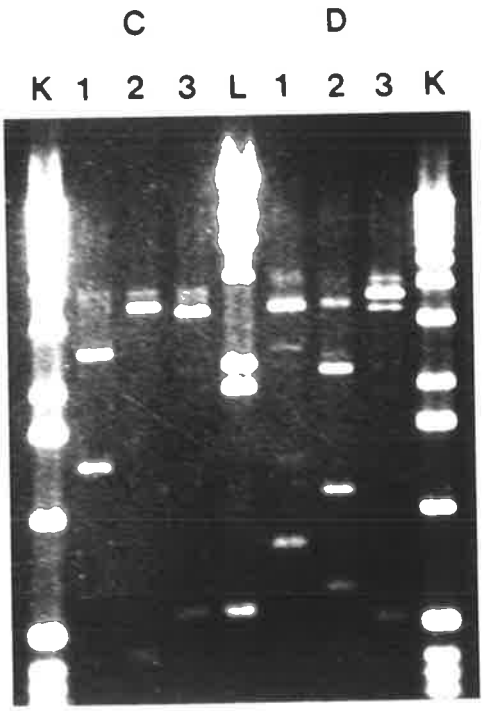


Table 4.3 Analysis of complete *Bam*HI, *Hin*dIII and *Sma*I digests of pAgK1318 by hybridization with DIG-labelled *Xba*I fragments.

Restriction enzyme	Fragment(s) hybridizing to DIG-labelled probes (kb)				
	10.5 kb	**4.3 kb**	**4.0 kb**	**2.3 kb**	**0.6 kb**
*Bam*HI	11.9	4.4	4.2	11.9	11.9
	7.0	9.1*	4.4*		
	4.2*				
HindIII	7.0	4.7	4.7	7.0	1.5
	3.4	2.8	0.6*	1.5	
	1.5	2.3			
*Sma*I	25	6.1	6.1	25	25
	8.5	9.5*	8.5*		

* weakly hybridizing

Table 4.4 Products from the digestion of pAgK1318 *Xba*I fragments with the restriction endonucleases *Bam*HI, *Hin*dIII and *Sma*I.

Restriction enzyme	Product(s) from the digestion of *Xba*I fragments (kb)				
	10.5 kb	**4.3 kb**	**4.0 kb**	**2.3 kb**	**0.6 kb**
*Bam*HI	7.0	3.8	3.4	2.3	0.6
	2.6	0.5	0.6		
	0.8				
*Hin*dIII	5.0	2.3	3.5	2.0	0.6
	3.4	1.2	0.4	0.3	
	1.5	0.7			
	0.2 (2)				
*Sma*I	7.2	3.4	2.7	2.3	0.6
	3.3	0.9	1.3		

Table 4.5 Summary of the Southern analysis of complete *Bam*HI, *Hin*dIII and *Sma*I digests of pAgK1318.

Restriction enzyme	Fragment size (kb)	Probe(s) hybridizing to fragment (kb)
*Bam*HI	14.5	*Xba*I-AB
	11.9	10.5, 2.3, 0.6, *Xba*I-AB
	9.1	4.3*, *Xba*I-AB
	7.5	*Xba*I-AB
	7.0	10.5
	6.7	*Xba*I-AB
	6.4	*Xba*I-AB
	6.0	*Xba*I-AB
	4.4a	4.3, 4.0*
	4.4b	*Xba*I-AB
	4.2	10.5, 4.0
	3.8	*Xba*I-AB
	2.7	*Xba*I-AB
	2.1	*Xba*I-AB
	1.3	*Xba*I-AB
	1.2	*Xba*I-AB
*Hin*dIII	11.8	*Xba*I-AB
	10.7	*Xba*I-AB
	8.5a,b	*Xba*I-AB
	7.8	*Xba*I-AB
	7.0	10.5, 2.3
	4.8	*Xba*I-AB

Table 4.5 Continued

Restriction enzyme	Fragment size (kb)	Probe(s) hybridizing to fragment (kb)
	4.7	4.3, 4.0
	3.5	*Xba*I-AB
	3.4a	10.5
	3.4b	*Xba*I-AB
	2.8	4.3, *Xba*I-AB
*Hin*dIII	2.4	*Xba*I-AB
	2.3a	4.3
	2.3b	*Xba*I-AB
	2.1	*Xba*I-AB
	1.5a	10.5
	1.5b	2.3, 0.6, *Xba*I-AB
	1.1	*Xba*I-AB
	0.6	4.0*
	25	2.3, 0.6, *Xba*I-AB
	13.0	*Xba*I-AB
	9.5	4.3*, *Xba*I-AB
	8.8	*Xba*I-AB
*Sma*I	8.5	10.5, 4.0*
	6.1	4.3, 4.0
	5.4	*Xba*I-AB
	1.6	*Xba*I-AB
	0.9	(not in gel transferred)

*weakly hybridizing

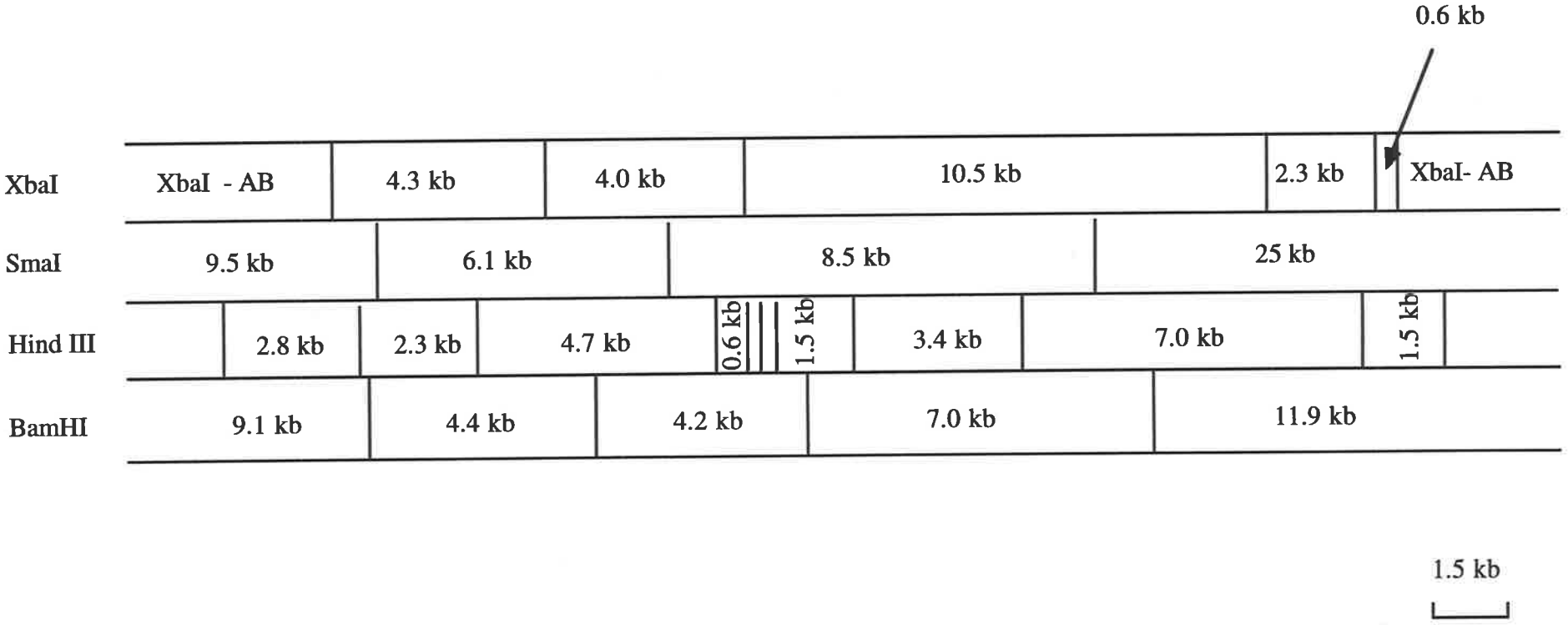
The map of the region of pAgK1318 inclusive of, and flanking, the novel 10.5 kb *Xba*I fragments with respect *Bam*HI, *Hin*dIII and *Sma*I is shown in Figure 4.7. From the map, it is possible to predict that on cutting *Xba*I-AB with *Hin*dIII a 2.1 kb fragment should be contiguous to the 4.3 kb *Xba*I fragment and a 0.6 kb fragment should be next to the 0.6 *Xba*I fragment. Earlier in the study, *Hin*dIII fragments of *Xba*I-AB were subcloned and recombinants with insert DNAs of sizes 2.1 and 0.6 kb with *Xba*I and *Hin*dIII ends were obtained.

In the map proposed, fragments smaller than 0.2 kb may have been excluded. Gels used for Southern analysis were run for extended periods to obtain better resolution of the larger fragments. Consequently, fragments up to 0.5 kb could have "run off" the gel. However, this should have been corrected for during multiple endonuclease digestions of the *Xba*I fragments as separations were carried out using various concentrations of agarose gel ranging from 1-2%.

The map can be used initially with a view to localizing the genes that may be involved in agrocin 434 synthesis. For example, reasonable-sized fragments in the vicinity of the deletion can be labelled and used to probe the existing *Hin*dIII fragment library of total K434 DNA to detect clones which will contain part or all of the region involved in agrocin 434 synthesis. Alternatively, such fragments can be cloned into a broad-host range vector and tested for production of nucleoside 4176, the biologically inactive fragment of agrocin 434. In addition, such clones can be used for complementation studies with strain K1318 to obtain production of agrocin 434.

Construction of the complete map of pAgK1318 should be facilitated by the availability of clones of the *Hin*dIII fragments of *Xba*I-AB. As mentioned earlier in the discussion, four distinct clones were obtained in which the insert DNA had a *Hin*dIII end and an *Xba*I end. These DNAs will be very useful as probes. The *Sma*I fragments of *Xba*I-AB can also be used as probes. Ordering of the fragments can then be carried out in a manner analogous to what had been done to generate a partial map of pAgK1318,

Figure 4.7 Map of the region of pAgK1318 inclusive of, and flanking, the novel 10.5 kb *Xba*I fragment with respect to the restriction endonucleases *Bam*HI, *Hin*dIII and *Sma*I.


0.6 kb
XbaI
XbaI - AB
4.3 kb
4.0 kb
10.5 kb
2.3 kb
XbaI- AB
SmaI
9.5 kb
6.1 kb
8.5 kb
25 kb
Hind III
2.8 kb
2.3 kb
4.7 kb
0.6 kb
1.5 kb
3.4 kb
7.0 kb
1.5 kb
BamHI
9.1 kb
4.4 kb
4.2 kb
7.0 kb
11.9 kb
1.5 kb

that is, mapping by a combination of multiple and partial endonuclease digestions and Southern analysis.

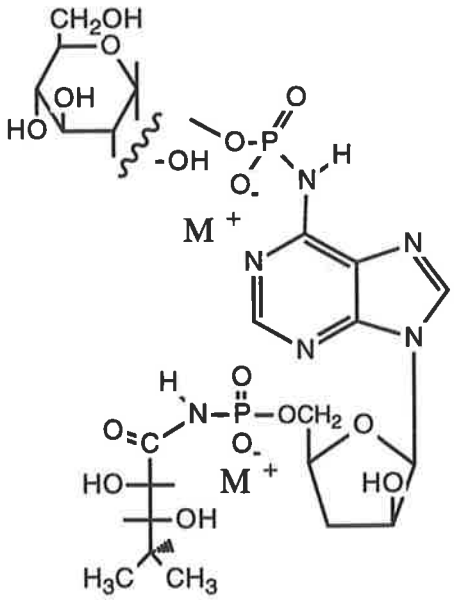
It has been suggested that the stepwise biosynthesis of antibiotics would require a dozen or more genes (Kerr and Tate, 1984). Experimental findings appear to bear this out. For example, it has been found that at least 15 kb of the *Pseudomonas fluorescens* HV37a genome is involved in the biosynthesis of oomycin A, the antibiotic implicated in the ability of this strain to prevent infection of cotton seedlings by *Pythium ultimum* (Gutterson, 1990). It has also been established that the biosynthesis of agrocin 84 is encoded on a contiguous 21-kb segment of pAgK84 (Ryder *et al.*, 1987; Wang *et al.*, 1994). Figure 4.8 shows that agrocin 434 is a disubstituted cytidine nucleoside. As such it is reasonable to predict that agrocin 434 may require less genetic information for its biosynthesis compared to the disubstituted adenine nucleotide agrocin 84 (Figure 4.8) which requires additional pathways for the synthesis of the fraudulent "core" nucleoside.

CONCLUSIONS

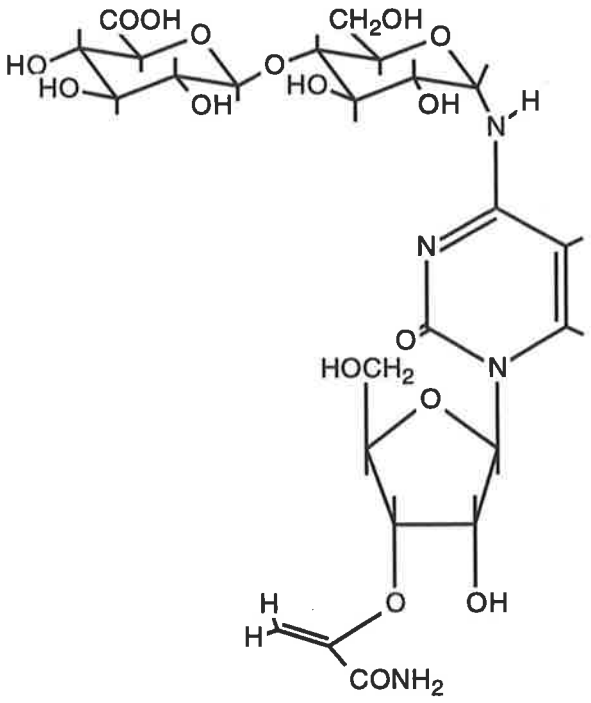
Good yields of high quality DNA were obtained from a procedure developed for the preparation and purification of plasmid DNA from *Agrobacterium* strain K1318, a K84 derivative carrying genes that encode synthesis of a biologically inactive fragment of agrocin 434. The procedure is simple, fast and very reliable.

A map of the region of pAgK1318 considered to contain some of the genes involved in the biosynthesis of agrocin 434 has been constructed using the restriction endonucleases *Bam*HI, *Hin*dIII, *Sma*I and *Xba*I. The map should prove useful in localizing genes involved in agrocin 434 synthesis and in studying other functions encoded on pAgK1318, and on the larger plasmid pAgK434.

Figure 4.8 The chemical structures of agrocin 84 and agrocin 434. Agrocin 84 is a disubstituted adenine nucleotide while agrocin 434 is a disubstituted cytidine nucleoside.



AGROCIN 84



Proposed AGROCIN 434

CHAPTER FIVE

AGROCIN 434: ITS URONIC ACID COMPONENT

INTRODUCTION

The name agrocin (Engler *et al.*, 1975) applies to low molecular weight antibiotic compounds produced by certain strains of *Agrobacterium*, which are inhibitory to other agrobacteria. A compound with these characteristics was first reported by Stonier (1960). Since then, a number of different agrocins have been reported (Kerr and Htay, 1974; Hendson *et al.*, 1983; Webster *et al.*, 1986; Chen and Xiang, 1986). The most well-characterized of these agrocins is the adenine nucleotide agrocin 84 (Kerr and Htay, 1974) because it is a major component in the biological control of crown gall by the non-pathogenic *Agrobacterium* strain K84 and its genetically-engineered derivative K1026 (Jones and Kerr, 1989; Ryder and Jones, 1990).

It has been found that both biocontrol strains produce another antibiotic called agrocin 434. This agrocin was first isolated from a K84 derivative strain, K434, from which it gets its name (Donner *et al.*, 1993). A provisional structure has been proposed for agrocin 434 as a disubstituted cytidine nucleoside (Donner and Tate, unpublished). The substituent at the N^4-position was described as a β-1,4-hexuronosyl-glucopyranosyl moiety. The nature of the uronic acid component has not been established.

The main aim of this study was to determine the nature of the uronic acid component of the agrocin 434 molecule. Other chemical aspects relevant to the elucidation of the complete chemical structure of the antibiotic have also been investigated.

MATERIALS AND METHODS

Bacterial Strains and Media

Agrobacterium strains K27, K1143 and K1318 used in this study have been described in Chapters 2 and 4. They were routinely maintained also as described in those two chapters.

Buffered glucose-glutamate (MMG) was used as the agrocin 434 production medium. Details of its preparation as well as of the other bacterial media employed in this study are given in Appendix A.

Chemicals

All solvents and solid reagents used, supplied mostly by Sigma and BDH Chemicals, were analytical grade or better.

Isolation and Purification of Agrocin 434

Isolation. Agrocin 434 was produced in the liquid medium MMG from strain K1143. The procedure developed in Chapter 3 was used for the production of agrocin 434 in 250-ml cultures. Before the agrocin was isolated, the cultures were incubated with shaking for 4-5 days at 28°C

The isolation protocol given in Chapter 2 was modified for the processing of 250-ml cultures. Using a GSA rotor, the cultures were centrifuged at 4°C (Sorvall Superspeed RC2-B centrifuge) for 10 min at 9000 rpm. The supernatant was decanted into clean centrifuge bottles. About 0.25 g activated charcoal was added to each bottle. The mixtures were left to adsorb for 30 min and then centrifuged for 10 min at 9000 rpm to pellet the charcoal. The samples were desalted by two successive washes with 200 ml distilled water. Agrocin 434 was recovered by elution in 30 ml 70% ethanol. The elution step was repeated twice.

The eluants were pooled and ethanol was removed using a rotary vacuum evaporator (Büchi EL 131 Rotavapor) with the water bath (Büchi 461 Water Bath) maintained at 32°C. The residue was dissolved in a minimum amount of 70% ethanol (about 20 ml), transferred to 2 ml tubes and centrifuged to remove the fine particles of charcoal that usually contaminated the residue. Ethanol was removed by drying under vacuum (SpeedVac SVC 100, Selby Scientific Instruments). The charcoal 70% ethanol desorbates were each dissolved in 20 µl of deionized water for further purification.

Purification. Agrocin 434 was purified by high voltage paper electrophoresis (HVPE) as described in Chapter 2. The buffer used for preparative HVPE was formic-acetic buffer, pH 1.75 (Appendix B) and separations were carried out at 3 kV for 15 min. Agrocin samples for chemical studies were separated using Whatman 20 Chr chromatography paper.

UV-absorbing bands were eluted from electrophoretograms by the procedure described in Chapter 2. The eluants were pooled and filtered through a 0.22 µm membrane filter (Millex®-GS) and then lyophilized. Samples were checked for biological activity by the bioassay procedure given in Chapter 2 using K27 as indicator strain. The UV absorption spectra of the samples were obtained using a Perkin Elmer Lambda 5 UV/Vis spectrophotometer.

Hydrolysis of Agrocin 434

Agrocin 434 samples were hydrolyzed with 1 M HCl for 3 hours at 110°C.

Borohydride Reduction

Portions of the agrocin 434 acid hydrolysate were reduced with sodium borohydride (20 mg/ml) for 30 min at room temperature.

Analysis of Hydrolysis and Reduction Products

Products from the hydrolysis and reduction of agrocin 434 were examined by HVPE as described in Chapter 2. The buffer systems used were formic-acetic acid buffer (pH 1.75), citrate buffer (pH 6.0), sodium tetraborate complexing buffer (pH 9.4) and ammonium bicarbonate buffer (pH 9.2). These buffers are further described in Appendix B. Separations carried out with formic-acetic buffer were usually run at 3 kV for 15 min while those with the other three buffers were done at 1.8 kV for 30 min. Longer runs were sometimes employed when greater resolution of components was desired.

The electrophoretic method of Miyamoto and Nagase (1981) for the separation of uronic acids from glycosaminoglycans was modified for this study. Chromatography paper (Whatman Chr 1) was used in place of Titan III cellulose acetate plates so that samples could be resolved using HVPE (Tate, 1968). In addition, formic-acetic acid buffer containing zinc acetate to a final concentration of 0.1 M was used in order to maintain constant pH in preference to the unbuffered 0.1 M zinc acetate.

Two sets of standards (Appendix B) consisting of various dyes, pyrimidine bases and nucleosides were run alongside samples.

Hydrolysis and reduction products were compared with authentic samples of sugars, sugar alcohols and uronic acids which included arabinose, glucose, ribose, xylose, glucitol, xylitol, galacturonic acid, glucuronic acid and glucuronolactone.

Staining

After examination with UV light, hydrolysis and reduction products were identified by staining the electrophoretograms using the methods of Trevelyan (1950) with some modifications.

The silver nitrate reagent (Appendix B) was used for the detection of vicinal diols. The paper strip was dipped in the silver nitrate reagent and allowed to dry. It was developed in a 2% ethanolic solution of NaOH (w/v), gently steamed and fixed with

thiosulfate reagent (10% sodium thiosulfate, 1.5% sodium metabisulfite). The paper was then soaked for at least 30 min in water to remove excess reagent and dried. A positive reaction was indicated by dark brown to black spots on a white background.

The presence of reducing sugars was detected by staining with tetrazolium reagent (Appendix B). The electrophoretogram was dipped in this reagent and the solvent allowed to dry. It was then developed in ethanolic NaOH (prepared as in silver staining), dried and steamed. Excess tetrazolium reagent was removed by soaking in water for at least 30 min. The paper strip was allowed to dry. The presence of a reducing sugar was indicated by an intensely red spot due to the formazan compound formed by the reduction of triphenyltetrazolium chloride in alkaline solution.

RESULTS AND DISCUSSION

5.1 Isolation and Purification of Agrocin 434

The procedure developed in this study for the large-scale production of agrocin 434 and the subsequent purification by HVPE gave an average yield of about 2 mg electrophoretically pure agrocin 434 per liter. Samples exhibited the characteristic UV absorption curve associated with agrocin 434 (Figure 5.1). This compound shows maximum absorption at 274 nm in aqueous solutions (pH $\approx$ 7) and a bathochromic shift to 288 nm on being made 0.1 M in HCl. The IR spectrum was recorded and compared with cytidine (Figure 5.2). The agrocin 434 spectrum is consistent with what would be expected of a molecule having a cytidine core.

Attempts to crystallize agrocin 434 from aqueous solutions for a confirmatory X-ray crystal structure determination, and as the hydrochloride from methanolic solutions using diethyl ether as precipitant have not been successful to date.

Figure 5.1 The ultraviolet (UV) spectrum of electrophoretically homogeneous agrocin 434 showing the characteristic absorption maximum at 274 nm in water (—) and the bathochromic shift to 288 nm in 0.1 M HCl (---).

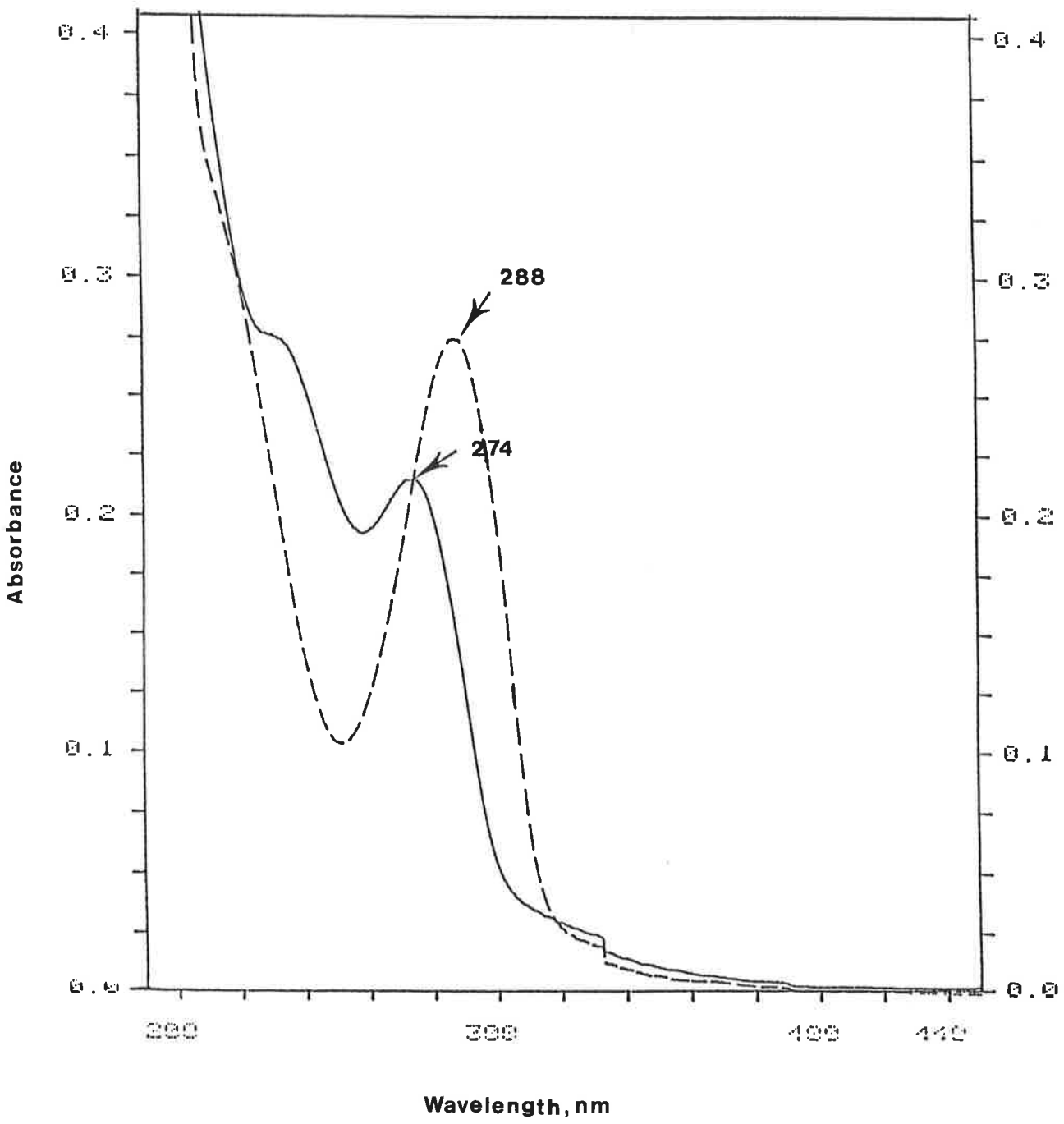
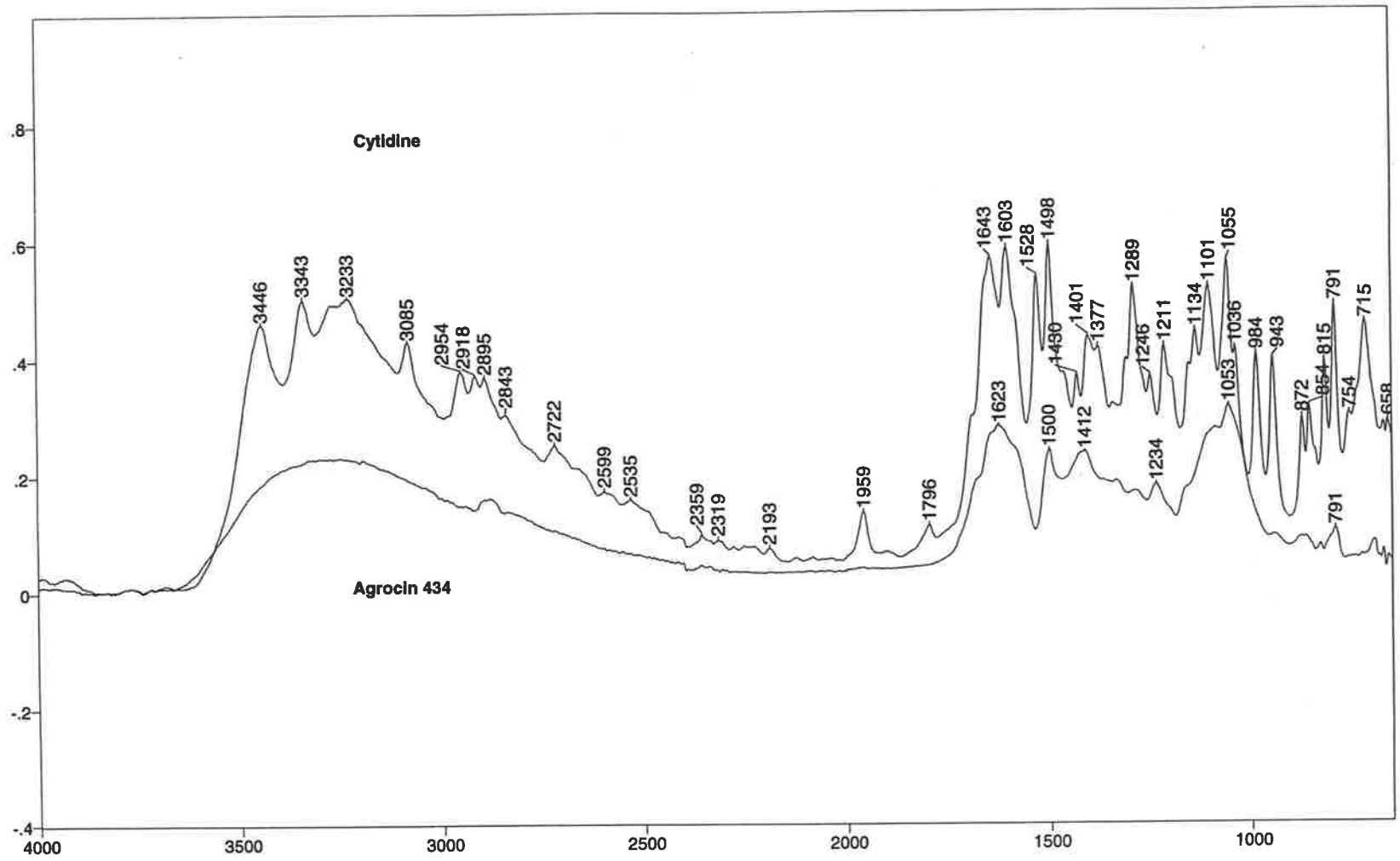

0.4
0.3
0.2
0.1
0.0
288
274
Absorbance
Wavelength, nm

Figure 5.2 The infrared (IR) spectrum of electrophoretically homogeneous agrocin 434 compared with that of cytidine.



Absorbance / Wavenumber (cm-1)

File # 2 : CYTIDINE

Overlay Y-Zoom CURSOR

5/9/95 5:38 PM Res=None

5.2 Identification of the Uronic Acid Component of Agrocin 434

The uronic acid component of the proposed aldobiouronic acid substituent at the N^4 position of cytidine was identified by a combination of degradative reactions and electrophoretic separations. Acid hydrolysis yielded components whose mobilities were indistinguishable from those of authentic samples of glucuronic acid and glucose, the other component of the proposed aldobiouronic acid moiety, when examined by HVPE (Table 5.1; Figure 5.3). In addition sodium borohydride reduction of the acid hydrolysate gave reduction products whose electrophoretic mobilities were indistinguishable from the corresponding reduction products of glucuronic acid and glucose (Table 5.1).

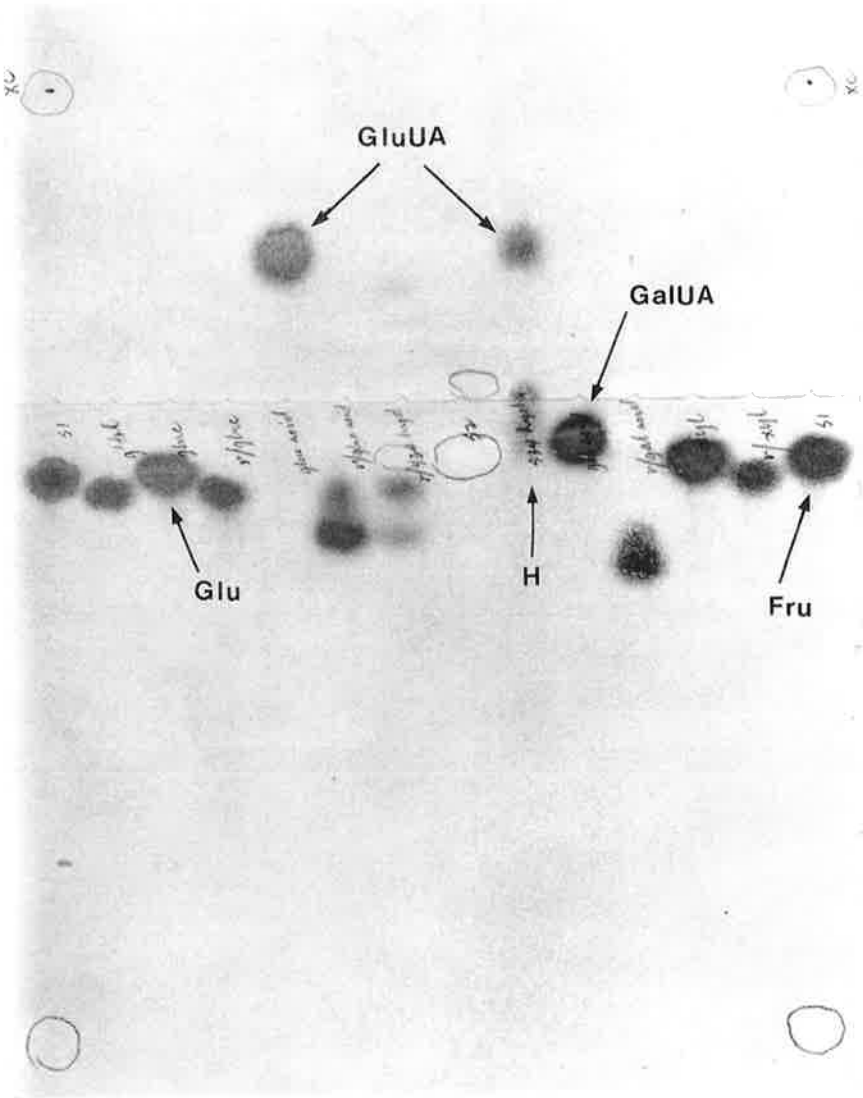
A third component of the agrocin 434 hydrolysate was detected with relative mobility values of 0.64 in borate buffer and 0.55 in ammonium bicarbonate buffer. These mobilities are what would be expected of the aldobiouronic acid moiety.

That the uronic acid component is glucuronic acid and not galacturonic acid was shown unequivocally in borate buffer and formic-acetic acid buffer containing 0.1 M zinc acetate as the two hexuronic acids were very well resolved in these complexing buffer systems (Table 5.1) in contrast to the poor separation in the non-complexing ammonium bicarbonate buffer.

The reduction products of the two uronic acids exhibited markedly different mobilities in formic-acetic acid buffer containing zinc acetate cation complexing buffer but not in the borate or bicarbonate buffer systems (Table 5.1).

Figure 5.3 Electrophoretogram showing comparison of agrocin 434 hydrolysis products (H) with authentic samples of glucuronic acid (GluUA), galacturonic acid (GalUA) and glucose (Glu). Fructose (Fru) was the zero mobility marker (RM=0). High voltage paper electrophoresis (Tate, 1968) was performed for 45 min at 1800 V.

(+)


GluUA
GalUA
Glu
H
Fru

(–)

Table 5.1 Relative electrophoretic mobilities of agrocin 434 degradation products using a zero mobility marker (RM_{OG} = 0.0) and a standard dyestuff, Orange G (RM_{OG} = 1.0).

Compound	RM_{OG}[a]			Stain[b]	
	1	2	3	A	B
Agrocin 434 hydrolysate	1.16	0.50	0.82	+	+
	0.96	0	0.55	+	+
	0.64	-	0	+	+
Glucuronic acid	1.16	0.48	0.82	+	+
Galacturonic acid	1.02	0.06	0.78	+	+
Glucose	0.96	0	0	+	+
Ribose	0.73	-	-	+	+
Reduction products					
Agrocin 434 hydrolysate	1.01	-0.06	0.81	+	−
(+ BH_4^-)	0.71	-0.17	0	+	−
Glucuronic acid (+ BH_4^-)	1.01	-0.06	0.84	+	−
		-0.17		+	−
Galacturonic acid (+ BH_4^-)	1.02	-0.24	0.78	+	−
Glucose (+ BH_4^-)	0.72	-0.06	0	+	−

[a] Buffer systems used: (1) 0.05 M sodium tetraborate, pH 9.4; (2) 0.75 M formic acid/1.03 M acetic acid/0.1 M zinc acetate; (3) 0.2 M ammonium bicarbonate/0.1 M ammonia, pH 9.2.

[b] Stains used: (A) Silver nitrate reagent for vicinal diols; (B) Tetrazolium reagent for reducing sugars.

In the absence of authentic samples, the distinction of glucuronic acid from other uronic acids was inferred from studies using the cationic complexing zinc acetate system which was the basis for the modified zinc-containing buffer, used in the current study, which enhanced the separation of glucuronic and galacturonic acids. Following on from the important work of St. Cyr (1970) who introduced the use of the zinc acetate complexing system (Table 5.2), Miyamoto and Nagase (1981) found that excellent electrophoretic separations of glucuronic acid from all other uronic acids could be achieved on Titan III cellulose plates using 0.1 M zinc acetate. In this system, they have demonstrated that glucuronic formed the weakest complex with the zinc cation and migrated furthest towards the anode (the positively-charged electrode). Galacturonic acid was found to have about the same mobility as iduronic acid indicating that the two formed the strongest complexes with zinc (Table 5.2).

In order to compare the efficiency of separation between glucuronic acid and galacturonic acid in buffered and unbuffered zinc acetate solutions, the two uronic acids were separated by HVPE (Tate, 1968). It was found that separation was better using buffered zinc acetate (Table 5.2). In addition, the large increases in current that can be expected from using unbuffered zinc acetate due to deposition of zinc hydroxide on the cathode were minimized by the use of the buffered system.

5.3 Determination of the Nature of Linkages

NMR data (Donner SC, unpublished) suggests the involvement of a β-1,4 linkage between the uronic acid and glucose in the agrocin 434 molecule. In an attempt to independently confirm this, agrocin 434 was treated with β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) which catalyzes the hydrolysis of β-glucuronides (Levvy and Conchie, 1966; Wakabayashi, 1970; Fishman, 1974). The attempt was unsuccessful. An extensive search of literature revealed that β-glucuronidase will only remove glucuronic acid residues when they are located in non-

reducing terminal positions of tetrasaccharides or larger fragments (Rodén, 1980). Neither the agrocin 434 molecule nor its disaccharide aldobiouronic fragment were substrates for this enzyme.

Table 5.2 Comparison of electrophoretic mobilities of hexuronic acids in 0.1 M zinc acetate (pH 6.2) and buffered 0.1 M zinc acetate (pH 3.0).

Hexuronic Acid	RM[a]			
	0.1 M Zinc Acetate			Buffered 0.1 M
	I[b]	II[c]	III[d]	Zinc Acetate[e]
Glucuronic acid	1.00	1.00	1.00	1.00
Mannuronic acid	0.75	–	–	–
Guluronic acid	0.44	–	–	–
Iduronic acid	0.32	0.29	–	–
Galacturonic acid	0.30	–	0.42	0.12

[a] Mobility relative to glucuronic acid; $RM_{glucuronic\ acid} = 1.00$.

[b] From St. Cyr (1970); Shandon electrophoresis apparatus, 600 V, 90 min.

[c] Calculated from Miyamoto and Nagase (1981) with correction for electroendoosmosis based on St. Cyr's (1970) mobilities; LKB Multiphor apparatus, 500 V, 30 min, 4°C.

[d] Tate's (1968) apparatus, 1800 V, 45 min.

Agrocin 434 samples have been subsequently analyzed by high field NMR spectrometry in the laboratory of Dr. Robert Brownlee at La Trobe University, Victoria.

Although minor impurities in the sample precluded the confirmation of all features of the proposed structure, the nature of the pyranosyl linkage between glucuronic acid and glucose was confirmed by Dr. Brownlee (personal communication) to be β-1,4. The aldobiouronic acid substituent is therefore cellobiouronic acid, present as an α–linked cellobiouronic moiety at position N^4 of the cytidine nucleoside as shown in Figure 5.4.

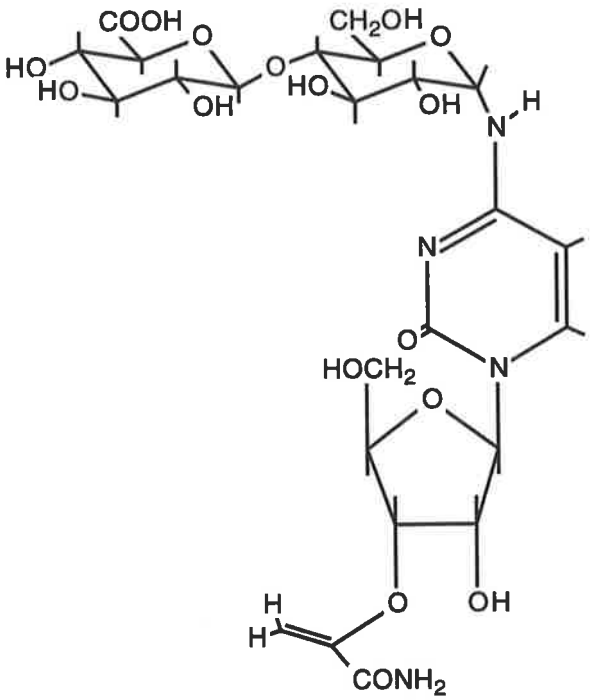
It is noteworthy that this same aldobiouronic unit was first reported as the repeating unit responsible for the rough exocellular polysaccharides of pneumococcus. The remarkable specificity exhibited by pneumococcus has been attributed to the chemical composition of the capsular polysaccharides (Goebel, 1935; Reeves and Goebel, 1941; Heidelberger and Rebers, 1958).

CONCLUSIONS

The current investigation has established that agrocin 434 contains a β-1,4-glucuronopyranosyl-α-1,N^4-glucopyranosyl substituent at the N^4 position of cytidine in the nucleoside as shown (Figure 5.4).

High voltage paper electrophoresis was found to be effective in the separation of uronic acids. The method is simple, rapid, inexpensive, and required no prior derivatization of the uronic acids. The modified zinc acetate buffer system, in particular, gave good resolution of the uronic acids and their reduction products using HVPE.

Figure 5.4 The proposed structure of agrocin 434.



Proposed AGROCIN 434

CHAPTER SIX

GENERAL DISCUSSION

There is much current interest in the use of biological agents to control the spread and severity of economically important crop diseases. The use of chemicals, which is the norm at the present, is expensive. In addition, there has been a growing awareness of the deleterious effects of chemicals on the environment and the development of pathogen resistance to chemical agents. The result has been an interest in finding microbial antagonists which may be used as alternatives to, or in combination with, agricultural chemicals.

The biological control of crown gall using strain K84 has been successfully practiced in many parts of the world since its introduction more than twenty years ago. A transfer-deficient derivative, K1026, was subsequently developed to safeguard the procedure from possible breakdown. K1026, as the pesticide product Nogall™, became the first ever genetically-engineered microorganism to be released for commercial application.

In both K84 and K1026, the production of an antibiotic compound known as agrocin 84, is thought to play a major role in the control of certain crown gall pathogens. Strong support for this hypothesis comes from the work of Wang *et al.* (1994) in which they have demonstrated the expression *in planta* of genes encoding the biosynthesis of agrocin 84 (*agn*) genes. That other factors also contribute to the control process has been suggested by studies in which strain K84 was shown to be active against agrocin 84-resistant pathogens (Cooksey and Moore, 1982; Lopez *et al.*, 1989).

A second antibiotic compound, designated agrocin 434, has been isolated from the biocontrol strains (Donner *et al.*, 1993). This agrocin is also produced by derivative strains such as K434 and K1143 both of which do not produce agrocin 84. In

this study, it was shown that agrocin 434 inhibits, *in vitro* , a wide range of biovar 2 agrobacteria that included both agrocin 84-sensitive and -resistant strains. While agrocin 434 may be less inhibitory in plate bioassays as compared to agrocin 84, it nevertheless extends the potential range of pathogens controlled by K84 to agrocin 84-resistant biovar 2 pathogens.

An implication of the sensitivity of biovar 2 strains to agrocin 434 is that this property may, at least in part, be chromosomally encoded. In contrast, sensitivity to agrocin 84 is conferred on the pathogen by their possession of agrocinopine catabolic genes borne on their Ti-plasmids (Ellis and Murphy, 1981; Hayman and Farrand, 1988).

Genes involved in the biosynthesis of agrocin 434 have been located on what was previously known as the cryptic plasmid (300-400 kb) and the designation pAgK434 has been recommended in recognition of this function (Donner *et al.*, 1993). It can be inferred from the insensitivity of the agrocin 434 producer strains to inhibition by the agrocin that some immunity functions are encoded on the agrocinogenic plasmid. In this regard, pAgK434 is similar to pAgK84 which encodes both agrocin 84 synthetic and immunity functions (Ellis *et al.*, 1979; Farrand *et al.*, 1985; Ryder *et al.*, 1987; Wang *et al.*, 1994). The involvement of genes on pAgK434 in resistance to agrocin 434 has been demonstrated by transferring the plasmid to an agrocin 434-sensitive strain (NC McClure, unpublished). The resulting strain was resistant to agrocin 434 and had acquired the ability to produce it as well. However, the isolation of a plasmid-free derivative of K84, K1347 (McClure *et al.*, 1994), which is resistant to agrocin 434, suggests that some immunity functions may also reside on the chromosome.

Mapping pAgK434 was considered as one of the strategies that would facilitate genetic studies on agrocin 434 as a supplementary agent for the biological control of crown gall. However, mapping a plasmid the size of pAgK434, which is about 300-400 kb, could be both laborious and difficult. Fortunately, a strain carrying a deleted version of pAgK434, has been isolated (Jones and Rosewarne, unpublished). This strain, called K1318, produces a biologically inactive fragment of agrocin 434, designated nucleoside 4176 (Figure 4.1). The resident plasmid, pAgK1318, is much

smaller in size (80-90 kb) and the result of a single large deletion in pAgK434 (NC McClure, personal communication). It has been shown to encode functions involved in the synthesis of the inactive fragment of agrocin 434. On the assumption that genes involved in the synthesis of agrocin 434 are clustered in a manner analogous to the agrocin 84 synthesis genes (Ryder *et al.*, 1987; Wang *et al.*, 1994), mapping of pAgK1318 was undertaken. As the likelihood of locating some of the genes is greatest in the vicinity of the deletion, fine mapping of the region inclusive of, and flanking, a novel 10.5 kb *Xba*I fragment was carried out. The result is a physical map of a 21.7 kb segment of pAgK1318 with respect to the restriction endonucleases *Bam*HI, *Hin*dIII, *Sma*I and *Xba*I.

The map should facilitate subsequent mutational and functional studies relevant to agrocin 434. For example, Tn*5* mutagenesis can be used to target genes involved in the biosynthesis of nucleoside 4176. Loss of this function can be readily monitored by electrophoretic (HVPE) and spectrophotometric (UV absorption) techniques. Reasonable-sized fragments can be cloned into broad-host-range vectors to test for nucleoside 4176 production. Alternatively, DNA fragments within the mapped region can be labelled and used to probe the existing *Hin*dIII fragment library of total K434 DNA to isolate clones which will contain part or all of the agrocin 434 synthesis genes. As strain K1318 is resistant to agrocin 434, the map should also prove useful in localizing genes that encode immunity to agrocin 434. Finally, construction of the complete map of pAgK1318, and eventually of pAgK434, should be facilitated by this partial physical map and some of the clones generated in the process of constructing it.

The effect of media on the expression of sensitivity to agrocin 84 has been well-documented (Cooksey and Moore, 1980; Spiers, 1980; Dhanvantari, 1983; van Zyl *et al.*, 1986). The same was found to be true for the expression of sensitivity to agrocin 434. Whether this is due to non-production/inactivation of agrocin 434 on the particular medium, or to changes in the test strains as a result of growth on the medium remains to be clarified. This part of the study has reiterated the importance of instituting proper controls in the conduct of comparative studies in different laboratories.

Specifically, it emphasizes the importance of using a sufficiently buffered medium in *in vitro* assays when investigating aspects of inhibition not related to pH effects. Because pH changes exert a profound influence on many metabolic processes, adequate pH control is important for the correct interpretation of experimental data.

The inhibitory effect of agrocin 434 has been demonstrated not only in plate bioassays but also in leaf disc bioassays and stem inoculations with a variety of tomato and tobacco cultivars (McClure *et al.*, 1994). This study involved testing the biovar 2 pathogen K27 against a range of K84 derivative strains producing either agrocin 84, agrocin 434 as well as a strain (K1347) producing neither agrocin. Interestingly, there was no significant difference in callus prevention between K1143, an agrocin 434 producer, and K84 which produces both agrocins. Thus, a role for agrocin 434 in the overall biocontrol process is indicated. In these plant bioassays a clear reduction of gall formation by the pathogen was obtained with K1347. These results point to the involvement of other as yet unidentified factors in the demonstrable biological control by strains K84 and K1026.

For studies on agrocin 434, strain K1143 was employed as it has been shown to produce only agrocin 434. Growth of this strain and production of agrocin 434 in a buffered glucose-glutamate medium (MMG) was investigated. Agrocin 434 was produced throughout the growth cycle. In this respect it is similar to agrocin 84 whose production was shown to coincide with the rapid phase of growth (Formica, 1990). Consistent with Formica's observation, Wang *et al.* (1994) have demonstrated that all *agn* loci were expressed at all growth stages. Thus, while the antibiotic activity of agrocin 84 and 434 classifies them as secondary metabolites (Vining, 1995; Wang *et al.*, 1979) their constitutive production suggests otherwise.

It was also observed that agrocin 434 production was not a result of carbon catabolite repression as production started well before glucose depletion in the medium. In contrast, the biosynthesis of the antibiotic oomycin A by *Pseudomonas fluorescens* HV37a has been found to be regulated by glucose (James and Gutterson, 1986; Gutterson *et al.*, 1988). It should be rewarding to extend the study of agrocin 434

production to include other carbon sources because of the influence of these compounds on the type and levels of antibiotics produced. Combined with the knowledge of the kind and relative amounts of other chemicals that may be present in the rhizosphere, the information could prove useful in predicting whether the antibiotic will be produced *in situ* (Dowling and O'Gara, 1994).

The current investigation has established that agrocin 434 contains a β-1,4-glucuronopyranosyl-α–1,N^4-glucopyranosyl substituent at the N^4 position of cytidine in the nucleoside. Interestingly, agrocin 434 shares common features in its chemical structure with agrocin 84 (Figure 4.8). Both molecules have modified 3' nucleosides as core components and both have carbohydrate substituents which are essential for selective antibiotic activity (Murphy *et al.*, 1981). Removal of the glucuronic acid moiety from agrocin 434 yields a biologically inactive molecule (nucleoside 4176) produced by a mutant strain K1121 (Donner SC, unpublished) and its derivative strain K1318. Whether the glucuronic acid substituent is important for selective uptake or for toxicity remains to be elucidated. It seems likely that the role of glucuronic acid substituent has to do with selective uptake if a parallel can be drawn with agrocin 84. In this molecule, the glucose moiety is recognized by the permease whose proper substrate is agrocinopine A (Murphy, 1982; Kerr and Tate, 1984).

The nucleoside component of agrocin 434 could be responsible for the toxicity of the molecule because it may interfere in any of a number of steps involved in nucleic acid synthesis. But it may not be as simple as this. As Murphy *et al.* (1981) have demonstrated for agrocin 84, the fraudulent nucleoside moiety is not sufficient for inhibiting bacterial growth. Rather, it is the 5'-phosphoryl substituted nucleoside which is required for toxicity. The exact nature of the agrocin 434 moiety which is toxic to sensitive strains will have to be determined in similar structure-activity studies.

Another substituted cytidine nucleoside is agrocin 108 (Kerr and Tate, 1984). It was reported as being produced by a pathogenic strain , K108 (Kerr and Htay, 1974). No further details have been reported other than that it is a 3'-O-β-D-xylopyranosyl cytidine with a 5'-phosphodiester substituent. Hendson *et al.* (1983) have also reported

another agrocin, D286, which is active against agrocin 84-resistant strains just like agrocin 434. Agrocin D286 exhibits the characteristic adenine-N^6-phosphoramidate UV absorption maximum at 264 nm, with a shoulder at 270 nm (Roberts *et al.*, 1977; Hendson *et al.*, 1983). There are no further reports on structure-activity correlations regarding agrocin D286 but Kerr and Tate (1984) suggested that its wider host range may be related to the nature of the substituents to the adenine core.

It has been shown that genetic determinants for the production of agrocin 84 are located on a contiguous 21-kb span of pAgK84. Agrocin 84 has been shown to be a disubstituted adenine nucleotide while the proposed structure of agrocin 434 is that of a disubstituted cytidine nucleotide. Because the fraudulent "core" nucleoside of agrocin 84 probably requires more steps for its biosynthesis, it is reasonable to predict that the synthesis of agrocin 434 will involve a DNA encoding region of a lesser magnitude than 21 kb. Another piece of information that may be useful in genetic studies is that strain K1318 encodes a fragment of agrocin 434 lacking the uronic acid component. The implication is that genes involved in the transfer of the glucuronic acid moiety to the nucleoside core were deleted when the parent strain of K1318 was Tn*5* mutagenized. These genes possibly encode functions relevant to the enzyme β-glucuronopyranosyl transferase.

While the production of agrocin 434 *in situ* has not been demonstrated, this does not argue against a role for agrocin 434 in controlling agrocin 84-resistant strains. A number of studies have also suggested that efficient root colonization may be a factor in the control process. Farrand and Wang (1992), however, raised the need for defining the parameters involved in root colonization in order to critically assess its role in biocontrol. Vicedo *et al.* (1993) and Wang *et al.* (1994) have discussed the role of factors in addition to agrocin 84, including additional agrocins, in the biological control of agrocin 84-resistant pathogens. Dowling and O'Gara (1994), in their review of the metabolites of *Pseudomonas* involved in plant disease control, reported that there is a growing body of evidence supporting the theory that metabolite production gives the producing strain a selective advantage in the very competitive environment of the plant

rhizosphere. The success of strain K84 as a biological control agent can thus be better appreciated in terms of how it has adapted to its particular niche in the rhizosphere.

In summary, it has been shown that agrocin 434, produced by the biocontrol strains K84 and K1026 in addition to agrocin 84, is inhibitory to biovar 2 agrobacteria. A partial physical map of the region considered to contain some of the genes involved in the synthesis of agrocin 434 has been constructed. A buffered glucose-glutamate medium (MMG) has been shown to support the growth of K1143, a K84 derivative strain, and the production of agrocin 434 by this strain. Agrocin 434 production appears not to be regulated by glucose. Finally, it has been established that agrocin 434 contains a β-1,4-glucuronopyranosyl-α-1,N^4-glucopyranosyl substituent at the N^4 position of cytidine in the nucleoside.

The exact mode of action of agrocin 434 in inhibiting crown gall caused by biovar 2 pathogens will need to be addressed in future investigations. In addition , the genetic basis of agrocin 434 biosynthesis and immunity to its effect also need to be elucidated. Synthesis of the compound on the basis of its proposed structure and demonstration of biological activity of the synthesized product will constitute a final proof of structure. Colonization and competition need to be studied more intensively in order to define their exact roles in biological control by K84. When a thorough understanding of the factors that have contributed to the remarkable success of this microbial agent is achieved, the development of other biological control agents to equal its success may be more attainable.

REFERENCES

Akiyoshi DE, Klee H, Amasino RM, Nester EW and Gordon MP. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proceedings of the National Academy of Sciences USA **81:** 5994-5998.

Alconero R. 1980. Crown gall of peaches from Maryland, South Carolina, and Tennessee and problems with biological control. Plant Disease **64:** 835-838.

Ankenbauer RG and Nester EW. 1990. Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. Journal of Bacteriology **172:** 6442-6446.

Ashby AM, Watson MD, Loake GJ and Shaw CH. 1988. Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C^1 toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. Journal of Bacteriology **170:** 4181-4187.

Ashby AM, Watson MD and Shaw CH. 1987. A Ti-plasmid determined function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. FEMS Microbiology Letters **41:** 189-192.

Barker RF, Idler KB, Thompson DV and Kemp JD. 1983. Nucleotide sequence of the T-DNA region from *Agrobacterium tumefaciens* octopine Ti plasmid pTil5955. Plant Molecular Biology **2:** 335-350.

Barry GF, Rogers SG, Fraley RT and Brand L. 1984. Identification of a cloned cytokinin biosynthetic gene. Proceedings of the National Academy of Sciences USA **81:** 4776-4780.

Beauchamp CJ, Chilton WS, Dion P and Antoun H. 1990. Fungal catabolism of crown gall opines. Applied and Environmental Microbiology **56:** 150-155.

Beringer JE. 1974. R factor transfer in *Rhizobium leguminosarum.* The Journal of General Microbiology **84:** 188-198.

Binns AN and Thomashow MF. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. Annual Review of Microbiology **42:** 575-606.

Bouzar H and Jones JB. 1992. Distinction of biovar 2 strains of *Agrobacterium* from other chromosomal groups by differential acid production. Letters in Applied Microbiology **15:** 83-85.

Braun AC. 1982. A history of the crown gall problems, pp. 155-210 in: Molecular Biology of Plant Tumors. G Kahl and JS Schell, eds. Academic Press, New York.

Buchanan-Wollaston V, Passiatore JE and Cannon F. 1987. The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature **328:** 172-175.

Burr TJ and Katz BH. 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap and vineyard soil. Phytopathology **73:** 163-165.

Cangelosi GA, Ankenbauer RG and Nester EW. 1990. Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proceedings of the National Academy of Sciences USA **87:** 6708-6712.

Cangelosi GA, Martinetti G, Leigh JA, Lee CC, Theines C and Nester EW. 1989. Role of *Agrobacterium tumefaciens* ChvA protein in export of beta-1,2-glucan. Journal of Bacteriology **171:** 1609-1615.

Casse F, Boucher C, Julliot JS, Michel M and Dénarié J. 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. The Journal of General Microbiology **113:** 229-242.

Chen X and Xiang W. 1986. A strain of *Agrobacterium radiobacter* inhibits growth and gall formation by biotype 3 strains of *Agrobacterium tumefaciens* from grapevine. Acta Microbiologica Sinica **26:** 193-199.

Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP and Nester EW. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell **11:** 263-271.

Christie PJ, Ward JE, Winans SC and Nester EW. 1988. The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-strands. Journal of Bacteriology **170:** 2659-2667.

Citovsky V, De Vos G and Zambryski P. 1988. Single-stranded DNA binding protein encoded by the *virE* locus of *Agrobacterium tumefaciens*. Science **240:** 501-504.

Clare BG. 1990. *Agrobacterium* in plant disease, biological disease control and plant genetic engineering. Science Progress, Oxford **74:** 1-13.

Clare BG. 1993. *Agrobacterium*: biological plant disease control, pp. 129-146 in: Advanced Engineered Pesticides. L Kim, ed. Marcel Dekker, Inc., New York.

Clare BG. 1995. Agrocins, pp. 619-632 in: Genetics and Biochemistry of Antibiotic Production. LC Vining and C Stuttard, eds. Butterworth-Heinemann, Boston.

Clare BG and McClure NC. 1995. *Agrobacterium*. pp. 221-236 in: Pathogenesis and Host Specificity in Plant Diseases, Vol. I: Prokaryotes. US Singh, RP Singh and K Kohmoto, eds. Elsevier Science Ltd, Oxford.

Clare BG, Kerr A and Jones DA. 1990. Characteristics of the nopaline catabolic plasmid in *Agrobacterium* strains K84 and K1026 used for biological control of crown gall disease. Plasmid **23:** 126-137.

Cooksey DA and Moore LW. 1980 Biological control of crown gall with fungal and bacterial antagonists. Phytopathology **70:** 506-509.

Cooksey DA and Moore LW. 1982. Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. Phytopathology **72:** 919-921.

Danna KJ. 1980. Determination of fragment order through partial digests and multiple enzyme digests. Methods in Enzymology **65:** 449-467.

Das PK, Basu M and Chatterjee GC. 1978. Studies on the mode of action of agrocin 84. The Journal of Antibiotics **31:** 490-492.

De Cleene M and De Ley J. 1976. The host range of crown gall. The Botanical Review **42:** 389-466.

Dessaux Y, Petit A and Tempé J. 1992. Opines in *Agrobacterium* biology, pp. 109-136 in: Molecular Signals in Plant-Microbe Communications. DPS Verma, ed. CRC Press, Inc.

Dessaux Y, Petit A and Tempé J. 1993. Chemistry and biochemistry of opines, chemical mediators of parasitism. Phytochemistry **34:** 31-38.

Dhanvantari BN. 1983. Etiology of grape crown gall in Ontario. Canadian Journal of Botany **61:** 2641-2646.

Donner SC, Jones DA, McClure NC, Rosewarne GM, Tate ME, Kerr A, Fajardo NN and Clare BG. 1993. Agrocin 434, a new plasmid encoded agrocin from the biocontrol *Agrobacterium* strains K84 and K1026, which inhibits biovar 2 agrobacteria. Physiological and Molecular Plant Pathology **42:** 185-194.

Douglas CJ, Staneloni RJ, Rubin RA and Nester EW. 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. Journal of Bacteriology **161:** 850-860.

Dowling DN and O'Gara F. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. Trends in Biotechnology **12:** 133-141.

Dürrenberger F, Crameri A, Hohn B, Koukolíková-Nicola Z. 1989. Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proceedings of the National Academy of Sciences USA **86:** 9154-9158.

Ellis JG and Kerr A. 1979. Transfer of agrocin 84 production from strain 84 to pathogenic recipients: a comment on the previous paper. pp. 579-583 in: Soil-Borne Plant Pathogens. B Schippers and W Gams, eds. Academic Press, London.

Ellis JG and Murphy PJ. 1981. Four new opines from crown gall tumours – their detection and properties. Molecular and General Genetics **181:** 36-43.

Ellis JG, Kerr A, van Montagu M and Schell J. 1979. *Agrobacterium:* genetic studies on agrocin 84 production and the biological control of crown gall. Physiological Plant Pathology **15:** 311-319.

Engler G, Holsters M, Van Montagu M and Schell J. 1975. Agrocin 84 sensitivity: a plasmid determined property in *Agrobacterium tumefaciens*. Molecular and General Genetics **138:** 345-349.

Farrand SK. 1990. *Agrobacterium radiobacter* strain K84: a model biocontrol system, pp. 679-691 in: New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases. RR Baker and PE Dunn, eds. Wiley-Liss, New York.

Farrand SK and Wang C. 1992. Do we really understand crown gall control by *Agrobacterium radiobacter* strain K84?, pp. 287-293 in: Biological Control of Plant Diseases. EC Tjamos, GC Papavizas and RJ Cook, eds. Plenum Press, New York.

Farrand SK, Slota JE, Shim J-S and Kerr A. 1985. Tn*5* insertions in the agrocin 84 plasmid: the conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin 84 production. Plasmid **13:** 106-117.

Fishman WH. 1974. β-Glucuronidase. pp. 929-943 in: Methods of Enzymatic Analysis, Vol. 2, 2nd edition. HU Bergmeyer, ed. Academic Press, New York.

Formica JV. 1990. Conditions for the production of agrocin 84 by *Agrobacterium radiobacter* K84. Journal of Industrial Microbiology **5:** 33-44.

Goebel WF. 1935. Chemo-immunological studies on the soluble specific substance of pneumococcus. II. The chemical basis for the immunological relationship between the capsular polysaccharides of Types III and VIII pneumococcus. The Journal of Biological Chemistry **110:** 391-398.

Graves ACF and Goldman SL. 1986. The transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens*. Plant Molecular Biology **7:** 43-50.

Grimsley N, Hohn T, Davies JW and Hohn B. 1987. *Agrobacterium* mediated delivery of infectious maize streak virus into maize plants. Nature **325:** 177-179.

Gutterson N. 1990. Microbial fungicides: recent approaches to elucidating mechanisms. Critical Reviews in Biotechnology **10:** 69-91.

Gutterson N, Ziegle JS, Warren GJ and Layton TJ. 1988. Genetic determinants for catabolite induction of antibiotic biosynthesis in *Pseudomonas fluorescens* HV37a. Journal of Bacteriology **170:** 380-385.

Hayman GT and Farrand SK. 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. Journal of Bacteriology **170:** 1759-1767.

Hayman GT, von Bodman SB, Kim H, Jiang P and Farrand SK. 1993. Genetic analysis of the agrocinopine catabolic region of *Agrobacterium tumefaciens* Ti plasmid pTiC58, which encodes genes required for opine and agrocin 84 transport. Journal of Bacteriology **175:** 5575-5584.

Heidelberger M and Rebers PA. 1958. Cross reactions of polyglucose in antipneumococcal sera. VI. Precipitation of Type VIII and Type III antisera by β-glucans. Journal of the American Chemical Society **80:** 116-118.

Hendson M, Askjaer L, Thomson JA and van Montagu M. 1983. Broad-host-range agrocin of *Agrobacterium tumefaciens*. Applied and Environmental Microbiology **45:** 1526-1533.

Holmes B and Roberts P. 1981. The classification, identification and nomenclature of agrobacteria. The Journal of Applied Bacteriology **50:** 443-467.

Holsters M, Silva B, Van Vliet F, Genetello C, De Block M, Dhaese P, Depicker A, Inzé D, Engler G, Villaroel R, Van Montagu M and Schell J. 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid **3:** 212-230.

Hooykaas PJJ and Schilperoort RA. 1992. *Agrobacterium* and plant genetic engineering. Plant Molecular Biology **19:** 15-38.

Hooykaas-Van Slogteren GMS, Hooykaas PJJ and Schilperoort RA. 1984. Expression of Ti plasmid in monocotyledonous plants infected with *Agrobacterium tumefaciens*. Nature **311:** 763-764.

Htay K and Kerr A. 1974. Biological control of crown gall: seed and root inoculation. The Journal of Applied Bacteriology **37:** 525-530.

James Jr DW and Gutterson NI. 1986. Multiple antibiotics produced by *Pseudomonas fluorescens* HV37a and their differential regulation by glucose. Applied and Environmental Microbiology **52:** 1183-1189.

Jin S, Prusti RK, Roitsch T, Ankenbauer RG and Nester EW. 1990a. Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of *virG*. Journal of Bacteriology **172:** 4945-4950.

Jin S, Roitsch T, Ankenbauer RG, Gordon MP and Nester EW. 1990b. The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene regulation. Journal of Bacteriology **172:** 525-530.

Jones DA and Kerr A. 1989. *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. Plant Disease **73:** 15-18.

Jones DA, Ryder MH, Clare BG, Farrand SK and Kerr A. 1988. Construction of a Tra⁻ deletion mutant of pAgK84 to safeguard the biological control of crown gall. Molecular and General Genetics **212:** 207-214.

Jordan DC. 1984. Family III. Rhizobiaceae Conn 1938, pp. 234-256 in: Bergey's Manual of Systematic Bacteriology, vol. 1. NR Krieg and JG Holt, eds. Williams and Wilkins, Baltimore.

Kado CI. 1993. *Agrobacterium*-mediated transfer and stable incorporation of foreign genes in plants, pp. 243-254 in: Bacterial Conjugation. DB Clewell, ed. Plenum Press, New York.

Keane PJ, Kerr A and New PB. 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Australian Journal of Biological Sciences **23:** 585-595.

Kennedy BW and Alcorn SM. 1980. Estimates of U.S. crop losses due to procaryote plant pathogens. Plant Disease **64:** 674-676.

Kerr A. 1969. Transfer of virulence between isolates of *Agrobacterium*. Nature **223:** 1175-1176.

Kerr A. 1980. Biological control of crown gall through production of agrocin 84. Plant Disease **64:** 25-30.

Kerr A. 1982. Biological control of soil-borne microbial pathogens and nematodes, pp. 429-463 in: Advances in Agricultural Microbiology. NS Subba Rao, ed. Butterworth Scientific, London.

Kerr A. 1989. Commercial release of a genetically engineered bacterium for the control of crown gall. Agricultural Science **2:** 41-44.

Kerr A. 1992. The genus *Agrobacterium*, pp. 2214-2235 in: The Prokaryotes, 2nd edition. A Balows, HG Touper, M Dworkin, W Harder and KH Schleifer, eds. Springer-Verlag, New York.

Kerr A and Brisbane PG. 1983. *Agrobacterium*, pp. 27-43 in: Plant Bacterial Diseases. A Diagnostic Guide. PC Fahy and GJ Persley, eds. Academic Press, Australia.

Kerr A and Htay K. 1974. Biological control of crown gall through bacteriocin production. Physiological Plant Pathology **4:** 37-44.

Kerr A and Panagopoulos CG. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopathology **90:** 172-179.

Kerr A and Tate ME. 1984. Agrocins and the biological control of crown gall. Microbiological Sciences **1:** 1-4.

Kersters K and De Ley J. 1984. Genus III. *Agrobacterium*, pp. 244-254 in: Bergey's Manual of Systematic Bacteriology, vol. 1. NR Krieg and JG Holt, eds. Williams and Wilkins, Baltimore.

Kuldau GA, De Vos G, Owen J, McCaffrey G and Zambryski P. 1990. The *virB* operon of *Agrobacterium tumefaciens* pTiC58 encodes 11 open reading frames. Molecular and General Genetics **221:** 256-266.

Levvy GA and Conchie J. 1966. β-Glucuronidase and the hydrolysis of glucuronides. pp. 301-364 in: Glucuronic Acid, Free and Combined. GJ Dutton, ed. Academic Press, New York.

Liang YJ, Di YB, Zhoa JY and Ma DQ. 1990. Strain E26 of *Agrobacterium radiobacter* efficiently inhibits crown gall formation of grapevines. Chinese Science Bulletin **35:** 1055-1056.

Lippincott JA and Lippincott BB. 1975. The genus *Agrobacterium* and plant tumorigenesis. Annual Review of Microbiology **29:** 377-405.

López MM, Gorris MT, Salcedo CI, Montojo AM and Miró M. 1989. Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. Applied and Environmental Microbiology **55:** 741-746.

McCardell BA and Pootjes CF. 1976. Chemical nature of agrocin 84 and its effect on a virulent strain of *Agrobacterium tumefaciens*. Antimicrobial Agents and Chemotherapy **10:** 498-502.

McClure NC, Ahmadi AR and Clare BG. 1994. The role of agrocin 434 produced by *Agrobacterium* strain K84 and derivatives in the biological control of *Agrobacterium* biovar 2 pathogens. pp. 125-127 in: Improving Plant Productivity with Rhizosphere Bacteria. MH Ryder, PM Stephens and GD Bowen, eds. CSIRO Division of Soils, Adelaide.

Macrae S, Thomson JA and Van Staden J. 1988. Colonization of tomato plants by two agrocin-producing strains of *Agrobacterium tumefaciens*. Applied and Environmental Microbiology **54:** 3133-3137.

Marks JR, Lynch TJ, Karlinsey JE and Thomashow MF. 1987. *Agrobacterium tumefaciens* virulence locus *pscA* is related to the *Rhizobium meliloti exoC* locus. Journal of Bacteriology **169:** 5835-5837.

Matthee FN, Thomas AC and Du Plessis HJ. 1977. Biological control of crown gall. The Deciduous Fruit Grower **27:** 303-307.

Matthysse AG. 1987. Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. Journal of Bacteriology **169:** 313-323.

Miles PA and Misra SS. 1938. The estimation of the bactericidal power of blood. Journal of Hygiene **38:** 732-740.

Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Miyamoto I and Nagase S. 1981. A simple and rapid electrophoretic method for the separation of glucuronic acid and iduronic acid. Analytical Biochemistry **115:** 308-310.

Monod J. 1949. The growth of bacterial cultures. Annual Review of Microbiology **3:** 371-394.

Moore LW. 1977. Prevention of crown gall on Prunus roots by bacterial antagonists. Phytopathology **67:** 139-144.

Moore LW. 1979. Practical use and success of *Agrobacterium radiobacter* strain 84 for crown gall control. pp. 553-568 in: Soil-Borne Plant Pathogens. B Schippers and W Gams, eds. Academic Press, London.

Moore LW and Warren G. 1979. *Agrobacterium radiobacter* strain 84 and biological control of crown gall. Annual Review of Phytopathology **17:** 163-179.

Murphy PJ. 1982. Agrocin 84 selectivity and toxicity in *Agrobacterium*. PhD thesis, University of Adelaide, Adelaide, South Australia.

Murphy PJ and Roberts WP. 1979. A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. The Journal of General Microbiology **114:** 207-213.

Murphy PJ, Tate ME and Kerr A. 1981. Substituents at N^6 and C-5' control selective uptake and toxicity of the adenine-nucleotide bacteriocin, agrocin 84, in agrobacteria. European Journal of Biochemistry **115:** 539-543.

Nautiyal CS and Dion P. 1990. Characterization of the opine-utilizing microflora associated with samples of soil and plants. Applied and Environmental Microbiology **56:** 2576-2579.

New PB and Kerr A. 1972. Biological control of crown gall: field measurements and glasshouse experiments. The Journal of Applied Bacteriology **35:** 279-287.

Nixon BT, Ronson CW and Ausubel FM. 1986. Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntr*B and *ntr*C. Proceedings of the National Academy of Sciences USA **83:** 7850-7854.

Ophel K and Kerr A. 1987. Ecology of *Agrobacterium*: plasmids and biovars, pp. 3-5 in: Molecular Genetics of Plant-Microbe Interactions. DPS Verma and N Brisson, eds. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Ophel K and Kerr A. 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. International Journal of Systematic Bacteriology **40:** 236-241.

Panagopoulos CG, Psallidas PG and Alivizatos AS. 1979. Evidence of a breakdown in the effectiveness of biological control of crown gall, pp. 569-578 in: Soil-Borne Plant Pathogens. B Schippers and W Gams, eds. Academic Press, London.

Parke D, Ornston NL and Nester EW. 1987. Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. Journal of Bacteriology **169:** 5336-5338.

Parkinson JS and Kofoid EC. 1992. Communication modules in bacterial signaling proteins. Annual Review of Genetics **26:** 71-112.

Peralta EG, Hellmiss, R and Ream W. 1986. *Overdrive*, a T-DNA transmission enhancer on the *Agrobacterium tumefaciens* tumour-inducing plasmid. The EMBO Journal **5:** 1137-1142.

Pu X-A and Goodman RN. 1993. Tumor formation by *Agrobacterium tumefaciens* is suppressed by *Agrobacterium radiobacter* HLB-2 on grape plants. American Journal of Enology and Viticulture **44:** 249-254.

Reeves RE and Goebel WF. 1941. Chemoimmunological studies on the soluble specific substance of pneumococcus. V. The structure of the Type III polysaccharide. The Journal of Biological Chemistry **139:** 511-519.

Roberts WP, Tate ME and Kerr A. 1977. Agrocin 84 is a 6-N-phosphoramidate of an adenine nucleotide analogue. Nature **265:** 379-381.

Robertson JL, Holliday T and Matthysse AG. 1988. Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose synthesis and bacterial attachment. Journal of Bacteriology **170:** 1408-1411.

Rodén L. 1980. Structure and metabolism of connective tissue proteoglycans. pp. 267-371 in: The Biochemistry of Glycoproteins and Proteoglycans. WJ Lennarz, ed. Plenum Press, New York.

Rodriguez RL and Tait RC. 1983. Recombinant DNA techniques: an introduction. Addison-Wesley, Reading, Mass.

Ryder MH and Jones DA. 1990. Biological control of crown gall. pp. 45-63 in: Biological Control of Soil-Borne Plant Pathogens. D Hornby, ed. CAB International, Wallingford, UK.

Ryder MH, Slota JE, Scarim A and Farrand SK. 1987. Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. Journal of Bacteriology **169:** 4184-4189.

Ryder MH, Tate ME and Jones GP. 1984. Agrocinopine A, a tumor-inducing plasmid-coded enzyme product, is a phosphodiester of sucrose and L-arabinose. The Journal of Biological Chemistry **259:** 9704-9710.

St. Cyr. 1970. Electrophoresis of hexuronic acids. Journal of Chromatography **47:** 284-287.

Sambrook J, Fritsch EF and Maniatis T. 1989. Molecular Cloning. A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sawada H, Ieki H, Oyaizu H and Matsumoto S. 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. International Journal of Systematic Bacteriology **43:** 694-702.

Schroder G, Waffenschmidt S, Weiler EW and Schroder J. 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. European Journal of Biochemistry **138:** 388-392.

Schroth MN and Moller WJ. 1976. Crown gall controlled in the field with a nonpathogenic bacterium. Plant Disease Reporter **60:** 275-278.

Sciaky D, Montoya AL and Chilton M-D. 1978. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid **1:** 238-253.

Shaw CH, Ashby AM, Loake GJ and Watson MD. 1988. One small step: the missing link in crown gall. Oxford Surveys of Plant Molecular and Cell Biology **5:** 177-183.

Shim J-S, Farrand SK and Kerr A. 1987. Biological control of crown gall: construction and testing of new biocontrol agents. Phytopathology **77:** 463-466.

Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama M, Sakagami Y and Machida M. 1990. Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. Proceedings of the National Academy of Sciences USA **87:** 6684-6688.

Shirasu K, Morel P and Kado CI. 1990. Characterization of the *virB* operon of an *Agrobacterium tumefaciens* Ti plasmid: nucleotide sequence and protein analysis. Molecular Microbiology **4:** 1153-1163.

Slota JE and Farrand SK. 1982. Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. Plasmid **8:** 175-186.

Southern E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology **98:** 503-517.

Spiers AG. 1980. Biological control of *Agrobacterium* species *in vitro*. N.Z. Journal of Agricultural Research **23:** 133-137.

Stachel SE and Zambryski PC. 1986. *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46:** 325-333.

Stachel SE, Messens E, Van Montagu M and Zambryski P. 1985. Identification of the signal molecules produced by wounded plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. Nature **318:** 624-629.

Stachel SE, Timmerman B and Zambryski P. 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature **322:** 706-712.

Stonier T. 1956. Labeling crown gall bacteria with P^{32} for radioautography. Journal of Bacteriology **72:** 259-268.

Stonier T. 1960. *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. Journal of Bacteriology **79:** 889-898.

Tate ME. 1968. Separation of myoinositol pentaphosphates by moving paper electrophoresis (MPE). Analytical Biochemistry **23:** 141-149.

Tate ME, Murphy PJ, Roberts WP and Kerr A. 1979. Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. Nature **280:** 697-699.

Tempé J and Goldmann A. 1982. Occurrence and biosynthesis of opines, pp. 427-449 in: Molecular Biology of Plant Tumors. G Kahl and JS Schell, eds. Academic Press, New York.

Tempé J and Petit A. 1983. La piste des opines, pp. 14-32 in: Molecular Genetics of the Bacteria-Plant Interaction. A Pühler, ed. Springer-Verlag, Berlin.

Thomashow LS, Reeves S and Thomashow MF. 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. Proceedings of the National Academy of Sciences USA **81:** 5071-5075.

Thomashow MF, Hugly S, Buchholz WG and Thomashow LS. 1986. Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. Science **231:** 616-618.

Thomashow MF, Karlinsey JE, Marks JR and Hurlbert RE. 1987. Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. Journal of Bacteriology **169:** 3209-3216.

Tinland B, Hohn B and Puchta H. 1994. *Agrobacterium tumefaciens* transfers single-stranded tranferred DNA (T-DNA) into the plant cell nucleus. Proceedings of the National Academy of Sciences USA **91:** 8000-8004.

Toro N, Datta A, Carmi OA, Young C, Prusti RK and Nester EW. 1989. The *Agrobacterium tumefaciens virC1* gene product binds to overdrive, a T-DNA transfer enhancer. Journal of Bacteriology **171:** 6845-6849.

Trevelyan WE, Procter DP and Harrison JS. 1950. Detection of sugars on paper chromatograms. Nature **166:** 444-445.

Turk SCHJ, van Lange RP, Regensburg-Tuïnk TJG and Hooykaas PJJ. 1994. Localization of the VirA domain involved in acetosyringone-mediated *vir* gene induction in *Agrobacterium tumefaciens*. Plant Molecular Biology **25:** 899-907.

Van Haaren MJJ, Sedee NJA, Schilperoort RA and Hooykaas PJJ. 1987. Overdrive is a T-region enhancer which stimulates T-strand production in *Agrobacterium tumefaciens*. Nucleic Acids Research **15:** 8983-8997.

Van Larebeke N, Genetello C, Hernalsteens JP, De Picker A, Zaenen I, Messens E, van Montagu M and Schell J. 1977. Transfer of Ti plasmids between *Agrobacterium* strains by mobilization with the conjugative plasmid RP4. Molecular and General Genetics **152:** 119-124.

Van Larebeke N, Genetello C, Schell J, Schilperoort RA, Hermans AK, Hernalsteens JP and van Montagu M. 1975 Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature **255:** 742-743.

Van Onckelen H, Rudelsheim P, Hermans R, Horemans S, Messens E, Hernalsteens J-P, Van Montagu M and Greef JD. 1984. Kinetics of endogenous cytokinin, IAA and ABA levels in relation to the growth and morphology of tobacco crown gall tissue. Plant Cell Physiology **25:** 1017-1025.

Van Zyl FGH, Strijdom BW and Staphorst JL. 1986. Susceptibility of *Agrobacterium tumefaciens* strains to two agrocin-producing *Agrobacterium* strains. Applied and Environmental Microbiology **52:** 234-238.

Veluthambi K, Krishnan M, Gould JH, Smith RH and Gelvin SB. 1989. Opines stimulate induction of the *vir* genes of *Agrobacterium tumefaciens* Ti-plasmid. Journal of Bacteriology **171:** 3696-3703.

Vicedo B, Peñalver R, Asins MJ and López MM. 1993. Biological control of *Agrobacterium tumefaciens*, colonization, and pAgK84 transfer with *Agrobacterium radiobacter* K84 and the Tra$^-$ mutant strain K1026. Applied and Environmental Microbiology **59:** 309-315.

Vining LC. 1995. Genetic and environmental control of antibiotic production, pp. 1-7 in: Genetics and Biochemistry of Antibiotic Production. LC Vining and C Stuttard, eds. Butterworth-Heinemann, Boston.

Wakabayashi M. 1970. β-glucuronidases in metabolic hydrolysis. pp. 519-602 in: Metabolic Conjugation and Metabolic Hydrolysis, Vol. II. WH Fishman, ed. Academic Press, New York.

Wang C-L, Farrand SK and Hwang I. 1994. Organization and expression of the genes on pAgK84 that encode production of agrocin 84. Molecular Plant-Microbe Interactions **7:** 472-481.

Wang DIC, Cooney CL, Demain AL, Dunnill P, Humphrey AE and Lilly MD. 1979. Fermentation and Enzyme Technology. John Wiley and Sons, New York.

Wang K, Genetello C, Van Montagu M and Zambryski P. 1987. Sequence context of the T-DNA border repeat element determines its relative activity during T-DNA transfer to plant cells. Molecular and General Genetics **210:** 338-346.

Wang K, Herrera-Estrella L, van Montagu M and Zambryski P. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell **38:** 455-462.

Webster J, Dos Santos M and Thomson JA. 1986. Agrocin-producing *Agrobacterium tumefaciens* strain active against grapevine isolates. Applied and Environmental Microbiology **52:** 217-219.

Willmitzer L, De Beuckeleer M, Lemmers M, Van Montagu M and Schell J. 1980. DNA from Ti plasmid present in nucleus and absent from plastids of crown gall plant cells. Nature **287:** 359-361.

Willmitzer L, Schmalenbach W and Schell J. 1981. Transcription of T-DNA in octopine and nopaline crown gall tumours is inhibited by low concentrations of α-amanitin. Nucleic Acids Research **9:** 4801-4812.

Winans S. 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiological Reviews **56:** 12-31.

Yadav NS, Postle K, Saiki RK, Thomashow MF and Chilton M-D. 1980. T-DNA of a crown gall teratoma is covalently joined to host plant. Nature **287:** 458-461.

Yadav NS, Vanderleyden J, Bennett DR, Barnes WM and Chilton M-D. 1982. Short direct repeats flank the T-DNA on a nopaline Ti-plasmid. Proceedings of the National Academy of Sciences USA **79:** 6322-6326.

Yanisch-Perron C, Vieira J and Messing J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:** 103-119.

Yanofsky MF, Porter SG, Young C, Albright LM, Gordon MP and Nester EW. 1986. The *virD* operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. Cell **47:** 471-477.

Young JM, Takikawa Y, Gardan L and Stead DE. 1992. Changing concept in the taxonomy of plant pathogenic bacteria. Annual Review of Phytopathology **30:** 67-105.

Zaenen I, Van Larebeke N, Teuchy H, Van Montagu M and Schell J. 1974. Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. Journal of Molecular Biology **86:** 109-127.

Zambryski P. 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annual Review of Genetics **22:** 1-30.

Zambryski PC. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annual Review of Plant Physiology and Plant Molecular Biology **43:** 465-490.

Zambryski P, Depicker A, Krüger K and Goodman H. 1982. Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. Journal of Molecular and Applied Genetics **1:** 361-370.

Zorreguieta A, Geremia RA, Cavaignac S, Cangelosi GA, Nester EW and Ugalde RA. 1988. Identification of the product of an *Agrobacterium tumefaciens* chromosomal virulence gene. Molecular Plant-Microbe Interactions **1:** 121-127.

APPENDICES

APPENDIX A Bacterial Media and Antibiotics

H-Agar

H base plate, per liter

tryptone	10 g
NaCl	8 g
Bacto agar	12 g

H top agar, per liter

tryptone	10 g
NaCl	8 g
Bacto agar	7 g

Prepare base plates (approx. 20 ml per plate) with appropriate antibiotic. Overlay each plate with approximately 4 ml of top agar containing antibiotic, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropylthio-β-D-galactoside).

Per 40 ml top agar, add 160 µl X-gal (50 mg/ml; Promega) and 200 µl IPTG (100 mM; Sigma).

Luria Bertani (LB) broth, per liter

Tryptone	10
Yeast extract	5 g
NaCl	5 g

Adjust to pH 7.2 with NaOH.

For the preparation of plates, add 15 g agar per liter.

M-9 minimal medium, per liter

Na_2HPO_4	6 g
KH_2PO_4	3 g
NH_4Cl	1 g
NaCl	0.5 g
agarose	15 g

Add water to approximately 1 liter. Autoclave. Cool to 50°C. Add:

1 M $MgSO_4$	2 ml
1 M $CaCl_2$	0.1 ml
1 M thiamine-HCl	1 ml
20% (w/v) glucose	10 ml

MG medium, per liter

Mannitol	10 g
L-glutamic acid	2 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_20$	0.2 g
NaCl	0.2 g
Biotin	200 µg

Adjust to pH 7.0.

MMG medium, per liter

Sodium glutamate	1 g
Tri-sodium citrate	0.5 g
$(NH_4)_2SO_4$	1 g
K_2HPO_4	1.74 g
KH_2PO_4	5.44 g

Dissolve the solids in about 954 ml distilled water and autoclave. When cool, add the following filter-sterilized solutions:

0.5% $Fe(NO_3)_3$	1 ml
0.02% biotin	1 ml
1% thiamine	1 ml
Doy's trace elements *	1 ml
10% $MgSO_4$	2 ml
10% glucose	50 ml

Final pH is about 6.3.

* Doy's trace elements

$MnSO4 \cdot H_2O$	1 g
H_3BO_3	300 mg
$ZnSO_4 \cdot 7H_2O$	300 mg
$Na_2MoO_4 \cdot 2H_2O$	25 mg
$CuSO_4$	25 mg
$CoCl_2 \cdot 6H_2O$	25 mg

Dissolve solids and dilute to 100 ml with distilled water. Filter sterilize.

Nutrient broth, per liter

Nutrient broth (Oxoid)	13 g

Modified Stonier's medium, per liter

Tri-potassium citrate	10 g
Sodium glutamate	2 g
$CaSO_4$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
NH_4NO_3	2.7 g
NaH_2PO_4	0.3 g
K_2HPO_4	0.88 g
Fe-EDTA *	2.5 ml
$MnCl_2$ *	0.1 mg
$ZnCl_2$ *	0.5 mg
Biotin	200 µg
Agar	15 g
pH 7.0	

* Stock solutions:

Fe-EDTA – 278 mg $FeSO_4$ + 372 mg Na_2EDTA per 100 ml

$MnCl_2$ – 0.01 g per 100 ml

$ZnCl_2$ – 0.05 g per 100 ml

Dhanvantari's modification of Stonier's medium, per liter

Tri-potassium citrate	10 g
Sodium glutamate	2 g
L-glutamic acid	2 g
$CaSO_4$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
$Fe(NO_3)_3$	5 mg
$MnCl_2$	0.1 mg
$ZnCl_2$	0.5 mg
NaH_2PO_4	1.5 mg
K_2HPO_4	4.4 mg
Biotin	200 µg
Calcium pantothenate	10 mg
Nicotinic acid	10 mg
Agar	15 g

TY medium, per liter

Tryptone	5 g
Yeast extract	3 g
$CaCl_2 \cdot 6H_2O$	1.3 g
Agar	15 g

YM medium, per liter

Mannitol	10 g
Yeast extract	1 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
$FeCl_3$	0.01 g
$CaCl_2$	0.2 g
Agar	15 g
Adjust to pH 7.0.	

YEA medium, per liter

Nutrient broth	13.3 g
Yeast extract	1 g
Sucrose	5 g
$MgSO_4 \cdot 7H_2O$	0.24 g
Agar	15 g

For *A. rubi* the following growth factors are added per 100 ml YEA medium:

Biotin	100 µg
Calcium pantothenate	20 µg
Nicotinic acid	20 µg

YEB medium, per liter

Sucrose	5 g
Peptone	5 g
Beef extract	5 g
Yeast extract	1 g
1 M $MgSO_4$	2 ml
Adjust to pH 7.0	

Buffered soft agar, per 100 ml

0.2 M NaH_2PO_4	3.9 ml
0.2 M Na_2HPO_4	6.1 ml
Purified agar	0.7 g

Antibiotics

Ampicillin

Stock solution. 25 mg/ml of the sodium salt of ampicillin in water. Filter sterilize and store in aliquots at -20°C.

Working concentration. 50 µg/ml.

Kanamycin

Stock solution. 50 mg/ml of kanamycin monosulfate in water. Filter sterilize and store in aliquots at -20°C.

Working concentration. 200 µg/ml (for *Agrobacterium*)

APPENDIX B Miscellaneous Buffers and Solutions

Physiological Saline (Buffered Saline), per 100 ml

NaCl	0.85 g
0.2 M NaH_2PO_4	3.9 ml
0.2 M Na_2HPO_4	6.1 ml
pH 7.3	

Standard Markers

Set 1, per ml

2-deoxyadenosine	5 mg
Orange G	1 mg
Xylene cyanol	1 mg
Fructose	10 mg
Nitrobenzene sulfonate	4 mg
Sodium azide (preservative)	1 mg

Set 2, per ml

Cytosine	2 mg
Cytidine	2 mg
Uracil	2 mg
Uridine	2 mg
Nitrobenzene sulfonate	2 mg

HVPE Buffers

Ammonium Bicarbonate Buffer (0.2 M NH_4HCO_3/0.1 M NH_3; pH 9.2)

Dissolve 15.8 g NH_4HCO_3 (FW=79) and approximately 7 ml of 28-30% (w/w) NH_3 per liter solution.

Borate Buffer (0.05 M; pH 9.4)

Dissolve 19.07 g $Na_2B_4O_7 \cdot 10\ H_2O$ (FW=381.38) per liter solution.

Citrate Buffer (0.05 M; pH 6.0)

Dissolve 10.5 g citric acid (FW=210.14) and 5.7 g NaOH (FW=40) per liter solution.

Formic-Acetic Acid Buffer (0.75 M HCOOH/1.03 M CH_3COOH; pH 1.75)

Dissolve 28.4 ml 98% HCOOH and 59.2 ml glacial CH_3COOH per liter solution.

Formic-Acetic Acid Buffer containing 0.1 M Zinc Acetate (pH 3)

Dissolve 21.95 g $(CH_3COO)_2Zn \cdot 2\ H_2O$ (FW=219.49) and dilute to 1 liter with formic-acetic acid buffer.

Stains

Silver Nitrate Reagent, per liter

Dissolve 2 g $AgNO_3$ (FW=169.87) in 20 ml water. Dilute to 1 liter with acetone.

Tetrazolium Reagent, per 100 ml

Dissolve 0.2 g tetrazolium (2,3,5-triphenyl tetrazolium chloride; FW=334.8) in about 10 ml methanol. Dilute to 100 ml with chloroform.

APPENDIX C Publications Arising from this Thesis

Donner SC, Jones DA, McClure NC, Rosewarne GM, Tate ME, Kerr A, Fajardo NN and Clare BG. 1993. Agrocin 434, a new plasmid encoded agrocin from the biocontrol Agrobacterium strains K84 and K1026, which inhibits biovar 2 agrobacteria. Physiological and Molecular Plant Pathology **42:** 185-194.

Fajardo NN, Tate ME and Clare BG. 1994. Agrocin 434: an additional biological control component for crown gall, pp. 128-130 in: Improving Plant Productivity with Rhizosphere Bacteria. MH Ryder, PM Stephens and GD Bowen, eds. CSIRO Division of Soils, Adelaide.

APPENDIX D

ADDITIONAL DISCUSSION OF RECENT RELEVANT WORK

A recent publication by Penalver et al., (1994) described production of a new antibiotic-like substance, active *in vitro* against *A.tumefaciens.* This substance, designated ALS 84 was different from agrocin 84 and produced on mannitol-glutamate medium. Sensitivity was not determined by presence/absence of a Ti plasmid and ALS 84 had a bacteriostatic effect against a range of other bacterial genera. In this respect this proposed substance is also distinct from agrocin 434 which active exclusively against *A.rhizogenes* (biovar 2) pathogens. The effects of ALS 84 have only been observed *in vitro* and the chemical nature of this substance has not yet been elucidated. However it is important to consider the activity of this alternative product when interpreting the inhibition results observed during this study. In this study it has been suggested that the inhibition observed with certain biovar 3 strains was the result of a pH effect. There is also the possibility that additional inhibitory products such as ALS 84 may be responsible for this effect. Alternatively pH may affect the inhibitory activity of antibiotic-like products. These possibilities must be investigated further to explain the *in vitro* effects observed. In addition, the role these factors play in the biocontrol process must be examined with relevant *in planta* studies which are currently being conducted.

The description of agrocin 84 as an antibiotic or bacteriocin requires clarification. As agrocin 84 does not kill the producer cell it cannot be classed as a bacteriocin, therefore it is more correct to class agrocin 84 as an antibiotic even though it appears to be produced throughout the growth cycle, unlike many antibiotics which are secondary metabolites.

Vicedo et al., (1995) have also recently described studies into the transfer of plasmids pTi, pAtK84b and pAgK84 between *A.tumefaciens* and strain K84 in a plant tumour. Plasmid pAgK84b belongs to the Inc Rh1 incompatibility group and is thought ot act as a barrier to transfer of Ti plasmids of the same Inc group. However the work of Vicedo et al., (1995) has shown that strain K84 can act as a recipient for Inc Rh1 Ti plasmids and that in some cases pAgK84b is replaced whereas in other cases recombination has occured between the Ti plasmid and pAgK84b

As the largest fragments of pAgK1318 are indistinguisable in size it is possible to show the plasmid map obtained as a circular map, however the *Xba*I fragments A and B may be differentiated on size basis after further investigation.

Reference to the paper by Wang et al., (1994) also requires additional discusion. This paper showed that there were five *agr* transcriptional groups or operons, although several of these may be polycistronic so the exact number of genes is presently unknown. Although this paper showed that agrocin 84 genes are expressed *in planta* it did not provide proof for a role for agrocin 84 in the biological control process. The nature of the roles of the various agrocins in the biological process is being investigated in an another study (Ahmadi et al., 1995). Investigation into the role of carbon substrates in expression of *agn* genes showed that expression levels were observed in minimal media, regardless of the carbon source. In addition expression of each *agn* locus was not affected by the presence of other *agn* genes or the nopaline catabolic plasmid pAgK84b, opines or root exudates in the culture media. All five *agn* loci were expressed throughout the growth cycle.

Additional References

Ahmadi, A.R., McClure, N.C. and Clare, B.G. (1995). Factors involved in the bioogical control of crowngall disease by derivatives of *Agrobacterium rhizogenes* K84. Presented at "*Agrobacterium* and beyond" International conference, Gif sur Yvette, France, September 1995.

Penalver, R., Vicedo, B., Salcedo, C.I. and Lopez, M.M. (1994). *Agrobacterium radiobacter* strains K84, K1026 and K84 Agr$^-$ produce an antibiotic-like substance, active *in vitro* against *A.tumefaciens* and phytopathogenic *Erwinia* and *Pseudomonas* spp. Biocontrol Science and Technology 4 259-267.

Vicedo, B., Lopez, M.J., Llop, P. and lopez, M.M. (1995). Transfer of pTi, pAtK84b and pAgK84 plasmids between *Agrobacterium tumefaciens* and strain K84 of *A. radiobacter* in a tumour. Molecular characterisation of transferred plasmids. Presented at "*Agrobacterium* and beyond" International conference, Gif sur Yvette, France, September 1995.

Fajardo, N. N., Tate, M. E. & Clare, B. G. (1994). Agrocin 434: an additional biological control component for crown gall. In M.H. Ryder, P.M. Stephens and G.D. Bowen. (Eds.), *Improving plant productivity with rhizosphere bacteria: proceedings of the Third International Workshop on Plant Growth-Promoting Rhizobacteria, Adelaide, South Australia, March 7-11 1994.* (pp. 128-130). S. Aust., CSIRO Division of Soils.

Physiological and Molecular Plant Pathology (1993) **42**, 185–194

Agrocin 434, a new plasmid encoded agrocin from the biocontrol *Agrobacterium* strains K84 and K1026, which inhibits biovar 2 agrobacteria

S. C. Donner*, D. A. Jones†, N. C. McClure†, G. M. Rosewarne†, M. E. Tate*, A. Kerr†, N. N. Fajardo† and B. G. Clare†‡

**Plant Science Department and †Crop Protection Department, University of Adelaide, Waite Agricultural Research Institute, Glen Osmond, SA 5064, Australia*

(*Accepted for publication February 1993*)

Agrocin 434 is a previously unrecorded inhibitor produced by *Agrobacterium* strains K84 and K1026, which also secrete agrocin 84. These strains are used for the biological control of crown gall disease of plants and agrocin 434 may contribute to the biocontrol processes. When strain K84 was cured of the plasmids which encode agrocin 84 production (pAgK84) and nopaline catabolism (pAtK84b), the derivative strains retained both agrocin 434 production and the remaining 300–400 kb plasmid. This plasmid was mobilized into a plasmidless recipient and the resulting transconjugants also produced agrocin 434. Processes essential for biosynthesis of agrocin 434 are thus encoded on the large plasmid, for which the designation pAgK434 is recommended. Bioassays of the inhibitory effects of agrocin 434 were done with 68 *Agrobacterium* isolates from Australia, Europe and North America. All the biovar 2 (*A. rhizogenes*) isolates tested were inhibited, with the exception of the strains which produced agrocin 434 themselves. None of the biovar 1 (*A. tumefaciens*), biovar 3 (*A. vitis*), or *A. rubi* strains tested was sensitive to agrocin 434. Preliminary chemical analyses indicated that the compound is a di-substituted cytidine nucleoside.

INTRODUCTION

Pathogenic agrobacteria cause crown gall and hairy root diseases of a wide range of host plants and are responsible for significant economic losses [*4*, *20*]. Effective control of crown gall in a number of crops has been achieved in many countries through the use of the non-pathogenic biological control strain *Agrobacterium* K84, and its derivative K1026 [*15*, *16*, *18*]. Despite their wide-spread success, the biocontrol strains are not effective against all crown gall pathogens and they do not control all hairy root infections [*9*]. We are therefore attempting to develop strains with wider ranges of effectiveness. To this end we are investigating factors involved in the biocontrol processes.

Each of the biocontrol strains K84 and K1026 produces agrocin 84, which is specifically inhibitory to certain pathogenic agrobacteria in culture [*16*, *36*]. However, whilst it is accepted that agrocin 84 plays an essential part in controlling sensitive

‡To whom correspondence should be addressed.

Abbreviations used in text: Amp, ampicillin; Inc, incompatibility; Km, kanamycin; Nm, neomycin; Mob, mobilization; Rif, rifampicin; Str, streptomycin; Tc, tetracycline; Tn, transposon.

0885–5765/93/030185+10 $08.00/0

pathogens, it appears that *in planta* biocontrol also involves other factors. For instance, field studies by López *et al.* [*24*] have shown that both strain K84 and a mutant unable to produce agrocin 84 are able to reduce infection by some pathogenic agrobacteria which are insensitive to agrocin 84 in culture. These effects may in part be due to the superior root colonizing ability of the biocontrol strains [*31*], to the influence of other agrocins, or to as yet unidentified agents.

We have investigated the biological and chemical properties of additional inhibitory secretions from the biocontrol strains that may possibly contribute to the overall effect. Agrocin 434 is one such compound. It was detected initially as a product of strain K84 and is also produced by strain K434, a derivative of K84 lacking the ability to synthesize agrocin 84. The new agrocin was consequently named after strain K434. Although strain K434 is unable to produce agrocin 84 it nevertheless does have inhibitory activity *in vitro* against agrobacteria, including isolates insensitive to agrocin 84. Strain 434 has lost pAgK84, the 48 kb plasmid that encodes agrocin 84 synthesis, but retains the two other plasmids of its parent strain K84: pAtK84b, of 173 kb and bearing nopaline catabolic genes [*5*, *16*] and a 300–400 kb cryptic plasmid [*5*, *16*]. We present evidence that: (a) sensitivity to agrocin 434 is a characteristic of biovar 2 strains of *Agrobacterium* and; (b) that genes for the biosynthesis of this agrocin are encoded on the so-called cryptic plasmid. We propose that this plasmid be designated pAgK434 to indicate its agrocinogenic attribute: it is referred to as such throughout the paper.

MATERIALS AND METHODS

Bacterial strains

The biocontrol strain *Agrobacterium* K84, its agrocinogenic derivatives, the *Agrobacterium* and *E. coli* strains used to produce these derivatives, and their relevant plasmids are listed in Table 1.

The 68 *Agrobacterium* strains from Australia, Europe and North America, which were used in the survey, and their respective sensitivities to agrocin 434 are given in Table 2.

Bioassays for agrocin 434

We used standard agrocin bioassay methods [*21*], in which zones of inhibition were detected in lawns of sensitive strains overlaid on killed producer colonies. Where appropriate, the producer colonies were replaced by filter paper discs moistened with solutions of crude or partially purified agrocin recovered from 1·5–3·0 ml of liquid culture medium.

Agrocin 434 in liquid media: detection and isolation

Agrobacterium strains were grown for 2–3 days at 25 °C in a shake culture in a glucose-glutamate, liquid medium [*25*] modified to contain 1·74 g K_2HPO_4 and 5·44 g KH_2PO_4 per litre. Cells were removed by centrifugation and 1 ml samples of supernatant were mixed with 100 µl portions of an aqueous suspension of activated charcoal (15 mg ml^{-1}). The charcoal was washed three times by suspension and centrifugation in successive 1 ml volumes of water. After final centrifugation from water the charcoal was suspended in 200 µl of 70% ethanol, centrifuged and the

TABLE 1
Bacterial strains and plasmids used in agrocin 434 preparation and detection

Strain	Biovar*	Plasmids	Description
Agrobacterium			
K27	2	pTiK27 pAtK27	Indicator strain sensitive to both agrocins 84 and 434.
K84	2	pAgK84† pAtK84b‡ pAgK434§	Biological control strain, produces both agrocins 84 and 434.
K434	2	pAtK84b‡ pAgK434§	Spontaneous mutant of K84 lacking pAgK84, produces agrocin 434 but not agrocin 84.
K1143	2	pAgK434§	K434 cured of pAtK84b, produces agrocin 434 only; this study.
K749	1	—	C58 pTi⁻pAt⁻Rif^{r}Str^r, supplied by A. Kondorosi.
K1317	1	pAgK434§	K749 containing pAgK434 mobilized from K1143; this study.
Escherichia coli			
HB101		pJB3JI	Plasmid is a Km^s derivative of pR68.45, Tc^{r}Tra$^+$ [*2*].
HB101		pRK2013	Helper plasmid in triparental matings [*17*].
HB101		pUCD1000	Plasmid has Inc of pTiC58, Amp^r Km^r Nm^r [*12*].
S17–1		pSUP5011	Plasmid has Tn::Mob transposon, Km^r [*32*].

*For purposes of clarity, biovar designations are used rather than specific epithets.
†Agrocin 84 plasmid [*33*].
‡Nopaline catabolic plasmid [*5, 16*].
§Agrocin 434 plasmid [*5, 16* & this study].

supernatant collected. This procedure was repeated twice more and the combined 600 µl eluants were evaporated to dryness under vacuum. These crude extracts were each dissolved in 10 µl of de-ionized water for use in bioassays or in isolating agrocin 434.

Agrocin 434 was separated from crude extracts exhibiting inhibitory activity in bioassays, by high-voltage paper electrophoresis (HVPE), using the apparatus of Tate [*35*]. Samples of 10–50 µl were applied to filter paper strips which were immersed in either 1·03 M acetic/0·75 M formic acid, pH 1·75, 0·05 M citrate, pH 5–6, or sodium tetraborate, pH 9·4, and electrophoresis was carried out at 2–3 kV for 20–30 min. Prior to electrophoresis, samples of a solution containing the mobility reference compounds orange G, xylene cyanol and 2-deoxyadenosine [*7, 29*] were applied at the line of origin adjacent to the crude extract samples.

After electrophoresis the papers were dried and examined using transmitted UV illumination. Agrocin 434 activity was consistently associated with a UV-absorbent spot, found in inhibitory crude extracts, using each of the electrophoresis buffer systems.

For partial purification of agrocin 434, crude extracts were electrophoresed in formic/acetic acid. Discs containing the appropriate UV-absorbent spots were cut from dried electrophoresis paper strips and eluted in five successive 50 µl washes of

TABLE 2
In vitro *sensitivity of* Agrobacterium *strains to agrocin 434*

Strain	Pathogenicity*	Agrocin 434† response
Biovar 1‡		
A6, A358, Ach5§, B6, C58, K31	Ti	−
K64, K120, K198, K355, K549	Ti	−
NCPPB 396, NCPPB398, T37	Ti	−
K484, K578, K817, K820–K822	Ri	−
C58 (pTi^-), C58 (p^-)	O	−
Biovar 2		
Ag43, K27, K46, K71, K108	Ti	+
K114, K118, K440, K970	Ti	+
K744–K746	Ti	−
A4, ATCC 11325**, TR7	Ri	+
TR101, TR105	Ri	+
K84,K434,K1143	O	−
K47, K97, K103, K104	O	+
K123, K129, K130, K250	O	+
K251	O	+
Biovar 3		
Ag57, K309**, K374, K375–K377	Ti	−
K521, K995, K1070, K1072	Ti	−
A. rubi		
K864, K867, K868, K869, K872	Ti	−
K1047, ATCC13335(TR3)**	Ti	−

*O, non-pathogen; Ri, hairy root-inducing pathogen; Ti, tumour-inducing pathogen.
†+, inhibited by agrocin 434 *in vitro*; −, not inhibited by agrocin 434.
‡isolates of *A. rubi* are listed under this name but isolates of *A. tumefaciens*, *A. rhizogenes*, and *A. vitis*, are listed under their biovar designations since there appears to be more general agreement as to their meanings [*19*, *20*, *22*, *26*].
§Strains underlined were tested for their ability to synthesise agrocin 434 using bioassays and HVPE.
**ATCC 11325, ATCC 13335 (TR3) and K309 are type cultures of *A. rhizogenes*, *A. rubi*, and *A. vitis*, respectively [*26*].

de-ionized water. The solutions were either concentrated or dried under vacuum for storage. These partially purified preparations were used for bioassays and preliminary chemical studies.

Agrocin 434: preliminary chemical studies
Measurements were made of UV absorbance spectra of partially purified preparations dissolved in water, in 1 N HCl, and after hydrolysis in 3 M formic acid at 110 °C for 3 h.

Plasmid isolation and electrophoresis
Small scale plasmid preparations were done as described by Farrand *et al.* [*10*], for *Agrobacterium*, and by Sambrook *et al.* [*30*], for *Escherichia coli*. Either standard [*30*] or Eckhardt [*8*] methods for agarose gel electrophoresis were used.

Curing strain K434 of pAtK84b to produce strain K1143
Strain K434 has the two plasmids pAtK84b and pAgK434 (Table 1). It was cured of the former to produce a derivative having the single remaining plasmid pAgK434. Cosmid pUCD1000 carries the incompatibility function of pTiC58 [*12*] and hence is incompatible with pAtK84b in strain K434 [*5*]. Cosmid pUCD1000 was transferred from *E. coli* HB101 to K434 in a triparental mating with helper plasmid pRK2013 [*11*]. Km^r *Agrobacterium* colonies were then subcultured in a succession of liquid media, free of antibiotic. Km^s isolates were obtained which contained pAgK434, but which had lost both pAtK84b and the unstable pUCD1000, as determined by agarose gel electrophoresis. One of the resultant products was strain K1143 (Table 1).

Mobilization of pAgK434 from K1143 to K749
Tn*5-mob* was transferred from *E. coli* S17-1 (pSUP5011) to K1143 in a patch-mating on TY agar at 28 °C for 24 h. K1143 Km^r colonies were selected on Bergersen's medium [*1*] with 200 μg kanamycin per ml. These were patch-mated with HB101 (pJB3JI) on TY agar: Km^rTc^r K1143 colonies were selected on Bergersen's medium with kanamycin (200 μg ml^{-1}) and tetracycline (10 μg ml^{-1}), and patch-mated on TY agar with the plasmidless biovar 1 strain K749. Transconjugant K749 colonies were selected on biovar 1 selective medium [*19*, *20*] with kanamycin (200 μg ml^{-1}), and screened for plasmids in Eckhardt gels. Colonies with the single plasmid pAgK434 were tested for the complete range of biovar 1 characteristics [*26*]. Authentic biovar 1 strains were then tested for agrocin 434 synthetic capacity in bioassays using charcoal absorbed fractions of culture media, as described above, with K27 as the indicator strain. Inhibitory extracts were further purified by HVPE. UV absorbent spots from these extracts with the same electrophoretic mobility as agrocin 434 from strain K1143 were eluted from electrophoresis papers with de-ionized water and their UV absorbance spectra determined to ensure identity with agrocin 434. One of the biovar 1 transconjugant products was strain K1317 (Table 1).

RESULTS

Biovars and sensitivity to agrocin 434
All biovar 2 strains tested were inhibited by agrocin 434, from strain K1143, with the exception of K84 and its derivatives, and the three Ti-type pathogenic isolates (K744-K746) which proved to be agrocin 434 producers (Table 2).

In contrast, all biovar 1 and 3 and *A. rubi* strains tested were insensitive to agrocin 434 (Table 2). Four representative strains, from each of the biovar 1, 3 and *A. rubi* groups (Table 2), were tested for agrocin 434 production in liquid media and *in vitro* inhibitory capacity. None of the 12 strains was an agrocin 434 producer.

Association between pAgK434 and agrocin 434 production
Figure 1 (a) shows the plasmids of the parent biocontrol strain K84 and those of its two derivatives K434 and K1143, all of which have pAgK434. Figure 1 (b) shows the presence of pAgK434 in both the donor biovar 2 strain K1143 and K1317, one of the biovar 1 transconjugant products of a K1143 × K749 mating, and its absence from the plasmidless, biovar 1, recipient parent strain K749.

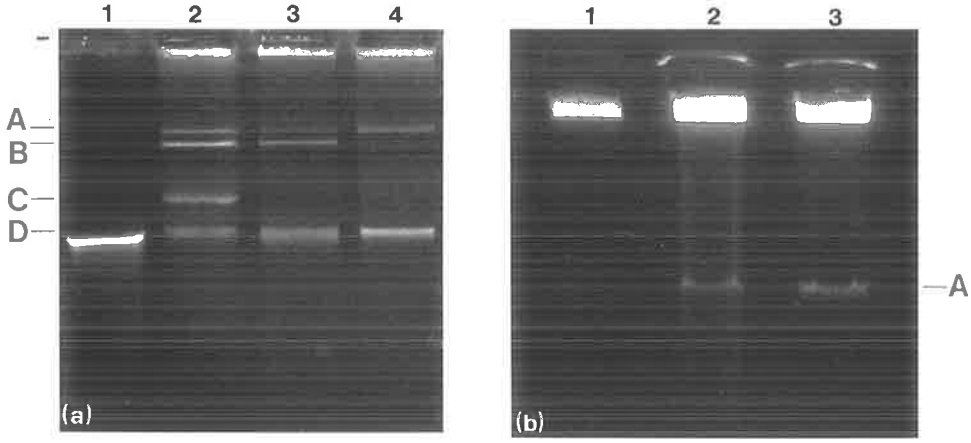


FIG. 1. Plasmids of *Agrobacterium* strains separated by electrophoresis in 0·7% agarose gel. (a) Lane 1, λDNA, 48 kb; lane 2, K84; lane 3, K434; lane 4, K1143. (b) Lane 1, K749; lane 2, K1317; lane 3, K1143. Band A, pAgK434; band B, pAtK84b; band C, pAgK84; band D, chromosomal DNA.

Agrocin 434 was produced by strains K84, K434, K1143, and K1317 but not by K749. Figure 2 shows the results of bioassays in which preparations from K1143 and K1317 produced zones of inhibition whereas K749 preparations did not. Figure 3 shows corresponding HVPE results demonstrating agrocin 434 presence in K1143 and K1317 secretions and its absence from those of K749. The same association between inhibitory action, agrocin 434 synthesis, and the presence of pAgK434 was recorded for a further 12 biovar 1 transconjugants produced by additional, independent K1143 × K749 matings.

Cultures of each of the pathogenic biovar 2 isolates K744–K746, which were insensitive to agrocin 434, produced a compound having the same inhibitory, electrophoretic and UV absorbance properties as agrocin 434 (data not shown). In addition, they each contained a plasmid of approximately the same size as pAgK434 as well as a pTi-sized plasmid (data not shown). The three isolates came from the one orchard and were indistinguishable in all of the cultural, biochemical, and plasmid characteristics examined: they thus appear to be identical isolates of the one strain.

Bioassay conditions

Colonies of strain K84, which secrete both agrocin 84 and agrocin 434, exhibited the characteristically transparent zones of inhibition associated with agrocin 84 [*15*, *21*]. In contrast, colonies secreting agrocin 434 alone produced opaque zones of inhibition that were smaller in diameter, much fainter, but still discernible. When discs impregnated with solutions of crude or partially purified agrocin 434 were used in bioassays, the zones of inhibition were more clearly visible (Fig. 2).

Agrocin 434: chemical data

The UV absorbance spectra in water and 1 N HCl (Fig. 4) were consistent with a ^{4}N substituted cytidine nucleoside. Formic acid hydrolysis yielded cytidine. Electrophoresis in borate buffer showed no increase in relative mobility over that in ammonium

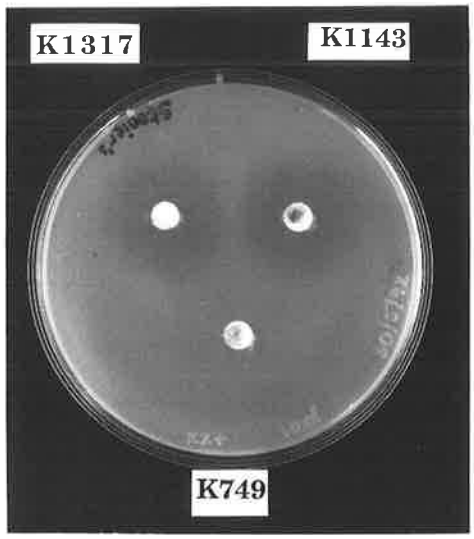


FIG. 2. Bioassay of charcoal adsorbed fractions from liquid media of cultures of the donor biovar 2 strain K1143, which contains pAgK434, the plasmidless biovar 1 recipient strain K749, and the resultant biovar 1 transconjugant K1317, which also contains pAgK434. Preparations from K1143 and K1317 both produced zones of inhibition but the K749 preparation was not inhibitory. K27 was used as the indicator strain.

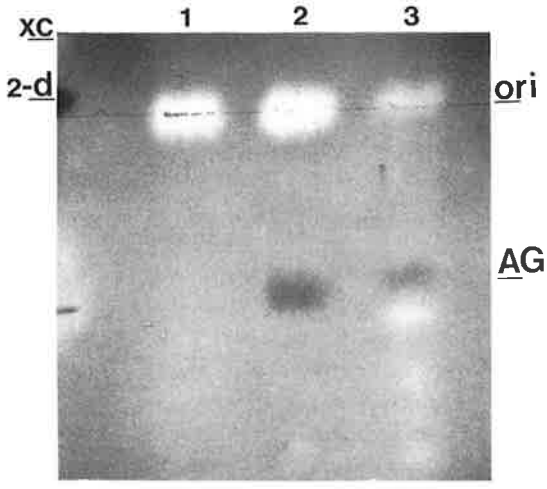


FIG. 3. High voltage paper electrophoresis of charcoal adsorbed fractions from liquid culture media. Electrophoresis was in 0·05 M citrate, pH 5·0, at 2 kV for 30 min: photographed using transmitted UV light. Lane 1, plasmidless strain K749; lanes 2 and 3, strains K1143 and K1317 respectively, each of which has the single plasmid pAgK434. AG indicates the positions of dark spots of UV-absorbent agrocin 434, which are present in the K1143 and K1317 lanes but absent from the K749 lane. Ori, origin for samples and mobility reference compounds; 2-d, 2-deoxyadenosine (UV absorbent) and xc, xylene cyanol (not visible in UV), indicate the positions to which these mobility reference compounds migrated. White spots are unidentified, UV-fluorescent compounds.

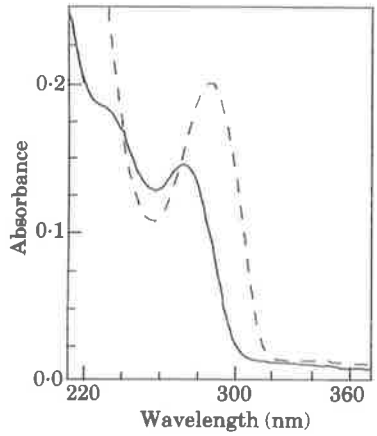


FIG. 4. UV spectra of partially purified agrocin 434 in H_2O (——) and 1 N HCl (------): absorbance maxima are 276 nm and 288 nm respectively.

carbonate, at the same pH. This establishes substitution at either position 2′ or 3′ of the ribose moeity. Hence agrocin 434 appears to be a disubstituted cytidine nucleoside.

DISCUSSION

Sensitivity to agrocin 434 was exhibited by all biovar 2 strains that were tested (Table 2), with the exceptions of wild-type or constructed agrocin 434 producer strains. The implication is that sensitivity to agrocin 434 is a general characteristic of biovar 2 strains and is presumably encoded chromosomally—at least in part. In contrast it has been shown that agrocin 84 sensitivity is exhibited only by pathogenic agrobacteria with *acc* genes borne on their Ti plasmids [*7*, *13*]. Another possible inference to be drawn from the insensitivity to agrocin 434 shown by agrocin 434 producer strains is that immunity to agrocin 434 is encoded on pAgK434. In this regard there may be a resemblance to pAgK84, which encodes both agrocin 84 immunity and synthesis [*28*].

None of the biovar 1 and 3 and *A. rubi* strains tested (Table 2) were inhibited by agrocin 434. A representative selection of these strains (Table 2) was shown not to produce agrocin 434. Hence their insensitivity to agrocin 434 appears to differ functionally from the immunity exhibited by agrocin 434 producers.

The broader effective inhibitory range of agrocin 434 in culture, when compared with agrocin 84, suggests that agrocin 434 may possibly be one of the factors contributing to *in planta* control by strain K84, and derivatives, of some *Agrobacterium* pathogens which are insensitive to agrocin 84 [*6*, *24*].

Our results indicate that essential steps in biosynthesis of agrocin 434 are encoded by pAgK434, the largest of the reported strain K84 plasmids. This conclusion is supported by the evidence that all K84 derivatives which contain this plasmid are agrocin 434 producers and that one of the agrocinogenic derivatives, K1143, has this plasmid only (Table 1). In addition, agrocin 434 synthesis was also exhibited by transconjugant biovar 1 strains, such as K1317 (Figs. 2 & 3), to which pAgK434 was transferred by conjugation [Fig. 1(b)]. Each of the pathogenic isolates (K744–K746) also has a

plasmid resembling pAgK434 in size, and each produces an inhibitor similar, or identical, to agrocin 434.

This is the first published record of a functional capacity for a plasmid of 300–400 kb, cryptic group found relatively commonly amongst agrobacteria. However an association has been reported previously between catabolism of deoxyfructosyl amino acids and the presence of the cryptic plasmid in a strain derived by curing strain C58 of its Ti plasmid [*27*]. These plasmids represent a substantial portion of the total genome of their host cells, and the identities of other functions encoded on them remains an intriguing question.

Our preliminary results on the chemistry of agrocin 434 indicate that it is a di-substituted cytidine nucleoside (Fig. 4). Since its appears to have a ^{4}N substitution it differs from agrocin 108 [*23*] and is also chemically distinct from the adenine-derived agrocins, 84 and D286 [*14*, *23*]. Agrocins have also been reported for strains H100 [*34*], HLB-2 [*3*], J73 [*37*], NCPPB 396 [*14*], and T37 [*34*], but since these compounds have not been characterized chemically it is not possible to make comparisons with agrocin 434.

This work was supported by grants from the Australian Research Grants Committee, the Australian Research Council and the Waite Institute Research Committee, and by scholarships from the Commonwealth Post-Graduate Research Scheme (S.C.D.) and the Australian International Development Assistance Bureau (N.N.F.). We thank Natalie Dillon for skilled technical assistance.

REFERENCES

1. **Bergersen FJ.** 1961. The growth of *Rhizobium* in synthetic media. *Australian Journal of Biological Sciences* **14**: 349–360.
2. **Brewin NJ, Beringer JE, Johnston AWB.** 1980. Plasmid-mediated transfer of host-range specificity between two strains of *Rhizobium leguminosarum*. *Journal of General Microbiology* **120**: 413–420.
3. **Chen XY, Xiang WN.** 1986. A strain of *Agrobacterium radiobacter* inhibits growth and gall formation by biovar III strains of *A. tumefaciens* from grapevine. *Acta Microbiologica Sinica* **26**: 193–199.
4. **Clare BG.** 1990. *Agrobacterium* in plant disease, biological disease control and plant genetic engineering. *Science Progress, Oxford* **74**: 1–13.
5. **Clare BG, Kerr A, Jones DA.** 1990. Characteristics of the nopaline catabolic plasmid in *Agrobacterium* strains K84 and K1026 used for biological control of crown gall disease. *Plasmid* **23**: 126–137.
6. **Cooksey DA, Moore LW.** 1982. Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. *Phytopathology* **72**: 919–921.
7. **Ellis JG, Murphy PJ.** 1981. Four new opines from crown gall tumours—their detection and properties. *Molecular and General Genetics* **181**: 36–43.
8. **Eckhardt T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1**: 584–588.
9. **Farrand SK.** 1990. *Agrobacterium radiobacter* strain K84: a model biocontrol system. In Baker RR, Dunn PE. eds *New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases*, New York: Wiley-Liss, 679–691.
10. **Farrand SK, Slota JE, Shim J-S, Kerr A.** 1985. Tn*5* insertions in the agrocin 84 plasmid: The conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin 84 production. *Plasmid* **13**: 106–117.
11. **Gallie DR, Hagiya M, Kado CI.** 1985. Analysis of *Agrobacterium tumefaciens* plasmid pTiC58 replication region with a novel high-copy-number derivative. *Journal of Bacteriology* **161**: 1034–1041.
12. **Gallie DR, Novak S, Kado CI.** 1985. Novel high- and low-copy stable cosmids for use in *Agrobacterium* and *Rhizobium*. *Plasmid* **14**: 171–175.
13. **Hayman GT, Farrand SK.** 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. *Journal of Bacteriology* **170**: 1759–1767.

14. **Hendson M, Askjaer L, Thomson JA, van Montagu M.** 1983. Broad-host-range agrocin of *Agrobacterium tumefaciens*. *Applied and Environmental Microbiology* **45**: 1526–1533.
15. **Jones DA, Kerr A.** 1989. *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. *Plant Disease* **73**: 15–18.
16. **Jones DA, Ryder MH, Clare BG, Farrand SK, Kerr A.** 1988. Construction of a Tra⁻ deletion mutant of pAgK84 to safeguard the biological control of crown gall. *Molecular and General Genetics* **212**: 207–214.
17. **Kao JC, Perry KL, Kado CI.** 1982. Indoleacetic acid complementation and its relation to host range specifying genes on the Ti plasmid of *Agrobacterium tumefaciens*. *Molecular and General Genetics* **188**: 425–433.
18. **Kerr A.** 1980. Biological control of crown gall through production of agrocin 84. *Plant Disease* **64**: 25–30.
19. **Kerr A.** 1992. The Genus *Agrobacterium*. In Balows A, Toüper HG, Dworkin M, Harder W, Schleifer KH, eds. *The Prokaryotes* Second edn. New York: Springer-Verlag, 2214–2235.
20. **Kerr A, Brisbane PG.** 1983. *Agrobacterium*. In Fahy PC, Persley GJ, eds. *Plant Bacterial Diseases. A Diagnostic Guide*. New York: Academic Press, 27–43.
21. **Kerr A, Htay K.** 1974. Biological control of crown gall through bacteriocin production. *Physiological Plant Pathology* **4**: 37–44.
22. **Kerr A, Panagopoulos CG.** 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathology* **90**: 172–179.
23. **Kerr A, Tate ME.** 1984. Agrocins and the biological control of crown gall. *Microbiological Sciences* **1**: 1–4.
24. **López MM, Gorris MT, Salcedo CI, Montojo AM, Miró M.** 1989. Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. *Applied and Environmental Microbiology* **55**: 741–746.
25. **Murphy PJ, Roberts WP.** 1979. A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. *Journal of General Microbiology* **114**: 207–213.
26. **Ophel K, Kerr A.** 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *International Journal of Systematic Bacteriology* **40**: 236–241.
27. **Ryder MH.** 1984. *Opines in Crown Gall and Hairy Root Diseases*. Ph.D. Thesis, University of Adelaide, South Australia.
28. **Ryder MH, Slota JE, Scarim A, Farrand SK.** 1987. Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. *Journal of Bacteriology* **169**: 4184–4189.
29. **Ryder MH, Tate ME, Jones GP.** 1984. Agrocinopine A, a tumor-inducing plasmid-coded enzyme product, is a phosphodiester of sucrose and L-arabinose. *The Journal of Biological Chemistry* **259**: 9704–9710.
30. **Sambrook J, Fritesch EF, Maniatis T.** 1989. *Molecular Cloning. A Laboratory Manual*, Second edn. Cold Spring Harbor, Cold Spring Harbor Laboratory Press: USA.
31. **Shim J-S, Farrand SK, Kerr A.** 1987. Biological control of crown gall: Construction and testing of new biocontrol agents. *Phytopathology* **77**: 463–466.
32. **Simon, R.** 1984. High frequency mobilisation of Gram-negative bacterial replicons by the *in vitro* constructed Tn*5*-mob transposon. *Molecular and General Genetics* **196**: 413–420.
33. **Slota JE, Farrand SK.** 1982. Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. *Plasmid* **8**: 175–186.
34. **Stonier T.** 1960. *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. *Journal of Bacteriology* **79**: 889–898.
35. **Tate ME.** 1968. Separation of myoinositol pentaphosphates by moving paper electrophoresis (MPE). *Analytical Biochemistry* **23**: 141–149.
36. **Tate ME, Murphy PJ, Roberts WP, Kerr A.** 1979. Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. *Nature* **280**: 697–699.
37. **Webster J, Dos Santos M, Thomson JA.** 1986. Agrocin-producing *Agrobacterium tumefaciens* strain active against grapevine isolates. *Applied and Environmental Microbiology* **52**: 217–219.