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THE AGROCIN ENCODING FUNCTION OF *AGROBACTERIUM TUMEFACIENS*

Mavis Hendson

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, for the Degree of Doctor of Philosophy.

Johannesburg, 1983.

DECLARATION

I declare that the thesis which is herewith submitted for the Doctor of Philosophy in the University of the Witwatersrand is my own unaided work except for the partial purification of agrocin D286. This part of the work was carried out by Lizzie Askjaer.

No part of this thesis has been previously submitted for a degree in any other university.

MHendson

Mavis Hendson

Johannesburg.
February, 1983.

ABSTRACT

Agrobacterium tumefaciens causes crown gall disease in dicotyledonous plants, reducing yield and therefore is a major economic problem. Biological control of this disease is desirable; other methods are costly or impractical. An agrocin producing *A. radiobacter* strain, K84, is used to control crown gall. However, many South African strains are resistant to K84. One aim of this study was to isolate new agrocin producing strains with a broader host spectrum. Another was to transfer the agrocin 84 encoding plasmid to a benign soil species, *Rhizobium*. One strain isolated, D286 (biotype 1), produced a broad host range agrocin active against strains carrying nopaline, octopine or agropine Ti plasmids. Agrocin was produced briefly in log phase. Strain D286 harboured a large cryptic plasmid and a smaller plasmid which may encode agrocin production.

Sensitivity to agrocin D286 was found to map at 11 - 18 Megadaltons in the nopaline Ti plasmid, pTiC58. The agrocin was partially purified, appeared to be a nucleotide, as is agrocin 84, and inhibited DNA, RNA and protein synthesis.

Plasmid RP4 was used to mobilise an agrocin 84 plasmid, pAt396, from *Agrobacterium* to *Rhizobium*. Co-transfer of pAt396 into *Agrobacterium* strains generally occured at a

low efficiency. Out of five thousand RP4-carrying *Rhizobium* transconjugants tested, only five produced agrocin. These transconjugants belonged to the species *Rhizobium meliloti*. They were, however, not able to inhibit gall formation by the virulent strain C58 on potato slices.

To my Parents

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ABBREVIATIONS

A_{540}	Absorbance at 540 nanometres
AU	Arbitrary unit
°C	Degrees Celsius
cpm	Counts per minute
D	Dalton
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetra-acetic acid
ery^R	Resistant to erythromycin
g	Gram
x g	Times gravity
hr	Hour
J	Joule
kan^R	Resistant to kanamycin
l	Litre
LA	Luria agar
LB	Luria broth
m	Metre
M	Molar
Md	Megadalton
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
MM	Minimal medium
nal^R	Resistant to naladixic acid
nm	Nanometre

no	Number
O D	Optical density
PPO	2,5 Diphenyloxazole
rif^R	Resistant to rifampicin
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
sec	Second
$strep/spec^R$	Resistant to streptomycin and and spectinomycin
TCA	Trichloroacetic acid
Ti	Tumor inducing
Tn	Transposon
Tris	Tris(hydroxymethyl)aminomethane
µCi	Microcurie
µg	Microgram
µl	Microlitre
UV	Ultraviolet
vol	Volume
V	Volt
wt	Weight

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1. GENERAL INTRODUCTION.

Agrobacterium tumefaciens is a Gram negative bacterium which has the unique capacity to induce neoplastic transformations, called crown galls in many species of dicotyledonous plants. Monocotyledonous plants are rarely, if ever, infected(Schell *et al.*, 1979; van Montagu and Schell, 1979; van Montagu *et. al.*, 1980). Once transformation has taken place, the infecting cells are no longer required for crown gall tumor maintenance. Crown gall tissues can be perpetuated in axenic culture in the absence of phytohormones, and tumors can be reinitiated in plants on which the tumor tissues are grafted(Braun and White, 1943; White, 1945; Braun, 1958; Braun and Wood, 1976; Braun, 1978).

In a survey of strains of Agrobacteria, Zaenen *et al.*(1974) demonstrated the presence of one or more large plasmids in a number of crown gall inducing *Agrobacterium* strains belonging to seven different *Agrobacterium* groups. These plasmids had molecular weights of 9×10^6 to 156×10^6 daltons. Plasmids were not found in eight non-pathogenic *Agrobacterium* strains belonging to four of the same groups. It was therefore hypothesized that the genetic information for the tumor inducing principle in crown gall

inducing *Agrobacterium* strains is carried by one or more large plasmids. It was firmly established(van Larebeke *et al*., 1974 and 1975; Watson *et al*., 1975; Bomhoff *et al*., 1976; Chilton *et al*., 1976; Currier and Nester, 1976; Kerr and Roberts, 1976; van Larebeke *et al*., 1977; Schell *et al*., 1979; van Montagu and Schell, 1979; van Montagu *et al*., 1980) by both physical and genetic evidence that the Ti plasmid is responsible for the induction of crown gall tumors. Virulence was stably lost when the bacteria were cured of the Ti plasmid by, for example, passage of the Ti bearing strain at elevated temperatures (37°C) (Hamilton and Fall, 1971; van Larebeke *et al*., 1974; Watson *et al*., 1975) and was acquired by avirulent strains when the plasmid was introduced by conjugation in plants(Kerr, 1969a; Kerr, 1971; van Larebeke *et al*.,1975; Watson *et al*., 1975; Kerr *et al*., 1977; Holsters *et al*., 1978c), *in vitro*(Bomhoff *et al*.,1976; Chilton *et al*., 1976; Kerr and Roberts, 1976; Levin *et al*., 1976; Genetello *et al*., 1977; Kerr *et al*., 1977; van Larebeke *et al*., 1977), or by transformation(Holsters *et al*., 1978a).

In contrast to the report by Zaenen *et al*.(1974), it was found that both oncogenic and non-oncogenic Agrobacteria contain large plasmids with molecular weights ranging between 90 and 182 x 10^6 daltons. Several strains contain more than one large plasmid

and in several cases only one of the plasmids is associated with oncogenicity(Currier and Nester, 1976; Merlo and Nester, 1977; Sciaky *et al.*, 1978). All oncogenic strains of *Agrobacterium tumefaciens* were found to carry a cryptic plasmid which is approximately 300 Md. No function has as yet been assigned to this plasmid.

Ti plasmid encoded functions can be determined by comparing wild type plasmids of *Agrobacterium tumefaciens* strains with mutant plasmids. Insertion and deletion mutants were generated by transposons(Hernalsteens *et al.*,1978; Dhese *et al.*, 1979; Holsters *et al.*, 1980; De Greve *et al.*, 1981). Restriction endonuclease and Southern blot analysis of wild type and mutant plasmids were carried out in order to localize these functions on the physical map of the Ti plasmids(Depicker *et al.*, 1980b; Holsters *et al.*, 1980; De Greve *et al.*,1981; De Vos *et al.*,1981). The functional maps of these plasmids are shown in Figure 1.1(Schell *et al.*, 1979; van Montagu and Schell, 1979; van Montagu *et al.*, 1980). In addition to containing a genetic component that specifies virulence, the Ti plasmid contains genetic information for the synthesis and utilization of certain unusual amino acids or opines(Lippincott *et al.*, 1973; Bomhoff *et al.*, 1976; Kemp, 1977; Montoya *et al.*,1977; Kemp *et al.*, 1979; Tempé *et al.*, 1979;

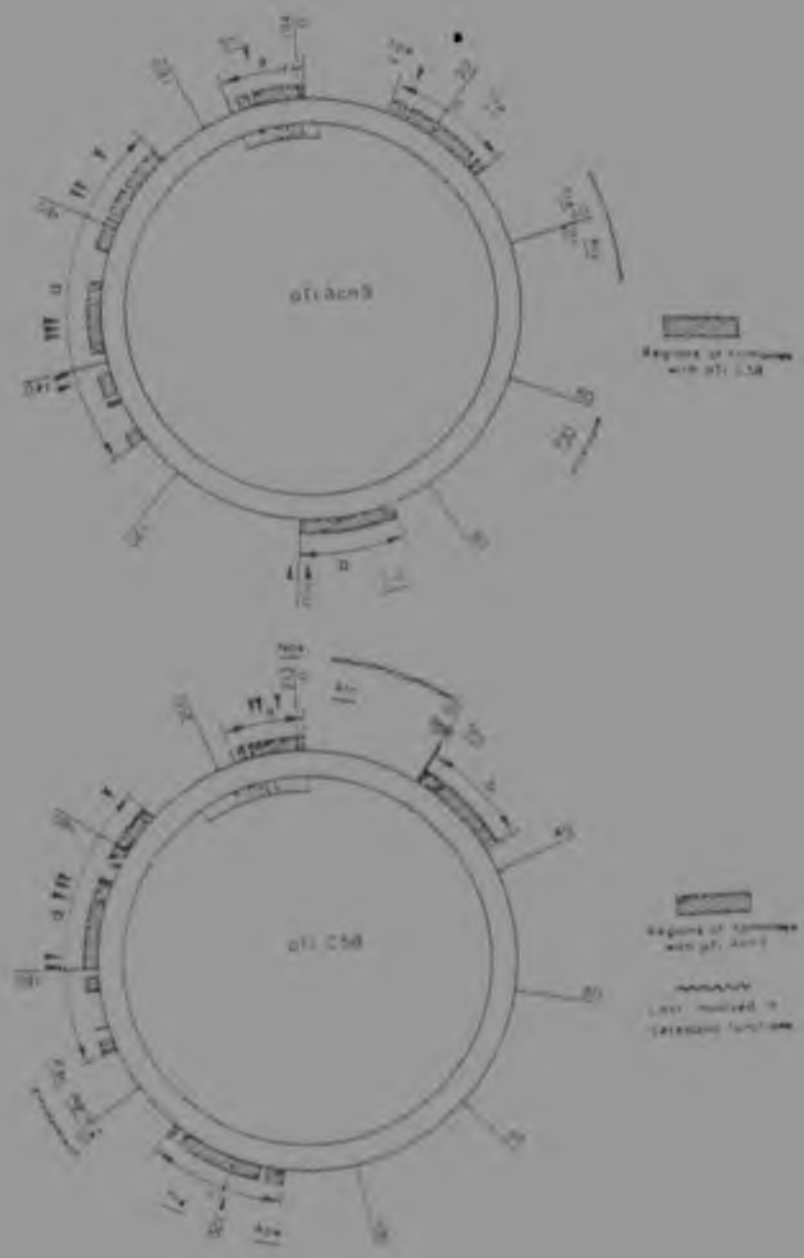


Figure 1.1 Functional map of a standard octopine (Ach5) and a nopaline (C58) Ti plasmid. The arrow heads indicate the sites where non-tumorigenic (Cnc) mutants were localised. The waved lines indicate the areas where functions involved in opine catabolism were mapped. The location of mutants in incompatibility (Inc), shoot induction (Shi), transfer function (Tra), phage exclusion (Ape), as well as biosynthesis of nopaline (Nos), catabolism of nopaline (Noc), of octopine (Occ), of arginine (Arc), or agropine (Agr), and of phosphorylated sugars (Psc), are shown (from van Montagu et al., 1980).

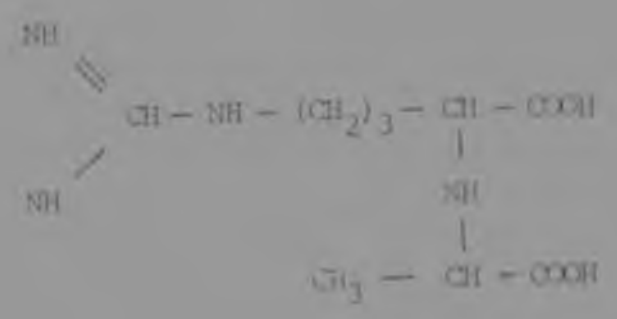
Schell *et al.*, 1979; van Montagu and Schell, 1979; van Montagu *et al.*, 1980). Hence, Ti plasmids are catabolic plasmids and have been arbitrarily classified into three types: octopine, nopaline and agropine types(Firmin and Fenwick, 1978; Tempé *et al.*, 1979; Guyon *et al.*, 1980).

The best studied of the opines are octopine, N^{α}-(D-1-carboxyethyl)-L-arginine, and nopaline, N^{α}-(1,3-dicarboxypropyl)-L-arginine(Tempé *et al.*,1979) derivatives of arginine which are not found in normal plant tissue(Kemp, 1976; Kemp *et al.*, 1979; Tempé *et al.*, 1979; Schell *et al.*, 1979; van Montagu and Schell, 1979; van Montagu *et al.*, 1980). The structure of octopine and nopaline are given in Figure 1.2. The type of arginine derivative synthesized by the tumor is determined by the bacterial strain which incites the tumor(Montoya *et al.*, 1977; Schell *et al.*, 1979; Tempé *et al.*, 1979). It is specified by genetic information on the virulence plasmid and is independent of the host plant on which the tumors are produced. Furthermore, it was demonstrated that the *Agrobacterium* strains that induced the synthesis of octopine in crown gall cells could selectively use octopine, but not nopaline, as sole energy, carbon and/or nitrogen source. Similarly, strains that induced the synthesis of nopaline in crown gall cells selectively

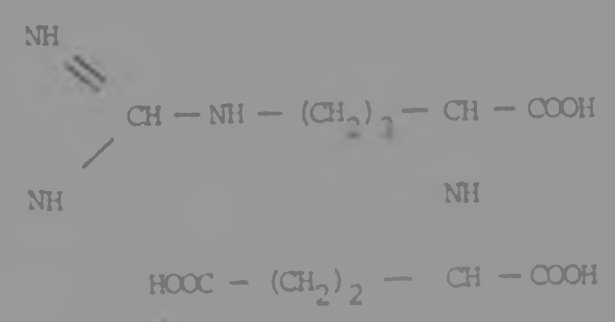
used it, and not octopine as sole energy, C and/or N source(Montoya *et al.*, 1977; Schell *et al.*, 1979; Tempé *et al.*, 1979; van Montagu and Schell, 1979; van Montagu *et al.*, 1980). Tumors that synthesize octopine also commonly synthesize octopinic acid, lysopine(Tempé *et al.*, 1979), histopine(Kemp, 1977) and agropine(Firmin and Fenwick, 1978). Nopaline tumors also contain nopalinic acid(Tempé *et al.*, 1979). "Null type" tumors metabolize the recently found opine with an N-polyol substituent, agropine(Firmin and Fenwick, 1978; Guyon *et al.*, 1980). The formula $C_{11}H_{17}NO_7$ has been assigned to this opine(Figure 1.2)(Coxon *et al.*, 1980).

The correlation between oncogenicity and opine metabolism has been confirmed by Lippincott *et al.*(1973), Kerr and Roberts(1976), Genetello *et al.*(1977) and Montoya *et al.*,(1977). However, since mutant plasmids can be isolated which fail to synthesize any opine, such synthesis is not necessary for tumor maintenance(Klapwijk *et al.*, 1976).

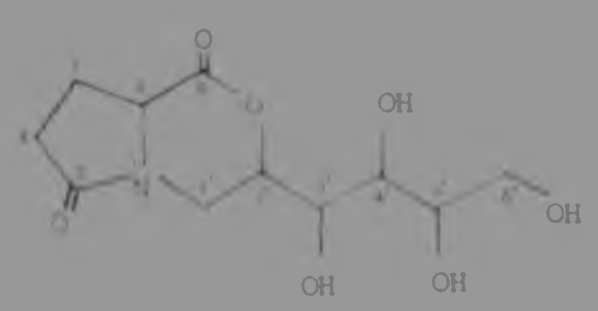
When *Agrobacterium tumefaciens* infects a plant it introduces genetic information into its host and induces the host to synthesize products coded for by the newly introduced genes, which it alone can utilize(Drummond, 1979; Schell and van Montagu, 1979; Schell *et al.*, 1979; van Montagu and Schell, 1979;



OCTOPINE



NOPALINE



AGROPINE

Figure 1.2 The structural formula of octopine, nopaline (from Tempé *et al.*, 1979) and agropine (from Coxon *et al.*, 1980)

van Montagu *et al*., 1980; Zambryski *et al*., 1980). The interaction of *Agrobacterium* with plants is, therefore, a novel sort of parasitism(Schell *et al*., 1979; Zambryski *et al*., 1980), which has been described as genetic colonization(Schell *et al*., 1979).

Ti plasmids have been found to be conjugative(Genetello *et al*., 1977; Kerr *et al*., 1977). The *tra* genes required for transfer are closely associated with opine metabolism, being inducible by the characteristic opine(Klapwijk *et al*., 1978; Petit *et al*., 1978; Hooykaas *et al*., 1979). Nopaline Ti plasmids synthesize two phosphorylated sugar derivatives called agrocinopine A and B(Ellis and Murphy, 1981), whereas agropine Ti plasmids synthesize agrocinopines C and D. In contradiction to previous reports, Ellis *et al*.(1982a) have shown that the opines agrocinopine A and B, and not nopaline promote the transfer of the nopaline Ti plasmid. Similarly, agrocinopines C and D promoted the transfer of agropine Ti plasmids(Ellis *et al*., 1982a). The *tra* functions map at 19-29 kilobases on the octopine Ti plasmid, pTiB6S3 (De Greve *et al*., 1981) and at 15-20 and 70-76.4 Megadaltons on the nopaline Ti plasmid, pTiC58(Holsters *et al*., 1980).

The tumorous state of crown gall cells has been demonstrated to be due to the transfer of part of the Ti plasmid, the T-DNA, to the plant cell. The extent and the arrangement of the T-DNA has been determined for several octopine and nopaline crown gall lines(Matthysse and Stump, 1976; Chilton *et al.*, 1977; Matthysse, 1977; Chilton *et al.*, 1978a; Schell *et al.*, 1979; van Montagu and Schell, 1979; Lemmers *et al.*, 1980; Merlo *et al.*, 1980; Thomashow *et al.*, 1980a; van Montagu *et al.*, 1980; Zambryski *et al.*, 1980). DNA was isolated from normal plant and crown gall tumor tissue (an octopine line), and was hybridised with different restriction endonuclease fragments of the *Agrobacterium* octopine plasmid(Chilton *et al.*,1977). Only two fragments were found to hybridise to tumor DNA but not normal plant DNA. About 20 copies of a 3.7 - 6 x 10^6 dalton plasmid segment were estimated to be present in each octopine tumor cell. Approximately 14-16 x 10^6 daltons of plasmid DNA was subsequently shown to be maintained in a nopaline tumor line, BT37(Yang *et al.*, 1980b).

The T-DNA has been shown to integrate stably into the plant genome(Thomashow *et al.*, 1980a; Yadav *et al.*, 1980; Zambryski *et al.*, 1980). Thomashow *et al.*(1980a) showed that all tumor lines contain a "core" T-DNA which is apparently responsible for

maintaining the transformed state. The core T-DNA is colinear with the Ti plasmid and contains the Ti plasmid sequences referred to as the "common DNA" - sequences which have been found in all Ti plasmids studied to date. It was also established that the Ti plasmid did not always give rise to the same T-DNA complement. Merlo *et al*.(1980) found that in three octopine tumor lines, a region of *Hin*d III fragment 1 of the octopine Ti plasmid was always present, and that the right hand boundary of T-DNA varied considerably among the three independent tumor lines. T-DNA encodes functions essential for oncogenicity since the insertion of RP4 into this portion of the nopaline Ti plasmid renders the Ti [illegible] non-oncogenic(Depicker *et al*., 1978). Genes on the T-DNA segment are involved in a number of functions, including plasmid transformation and opine biosynthesis(Thomashow *et al*., 1980a). Several parts of the T-DNA have been found not to be essential for tumor induction. Genes for the catabolism of the opines are not carried on the T-DNA(Holsters *et al*., 1980; De Greve *et al*., 1981).

An attempt was made to determine whether the T-DNA is transcribed in plants. RNA homologous to subfragments of T-DNA was observed in total RNA preparations of crown gall tissue(Drummond *et al*., 1977; Gurley *et al*., 1979; Yang *et al*., 1980a; Gelvin

et al., 1981). All T-DNA was found to be transcribed although different segments were transcribed to significantly different extents. The question of whether RNA transcribed from T-DNA actually codes for structural gene products is, however, still unresolved.

Hybridisation, electron microscopic(Currier and Nester, 1976; Engler et al., 1977; Depicker et al., 1978; Engler et al., 1981) and restriction endonuclease (fingerprint) studies of Ti plasmids(Depicker et al., 1977; Chilton et al.,1978b; Sciaky et al., 1978; Hepburn and Hindley, 1979; Engler et al., 1981) have shown that the plasmids of the octopine group are closely related to each other, while those in the nopaline group only show a limited degree of DNA sequence homology. For example, it has been shown by EcoRI fingerprinting(Genetello et al., 1977; De Greve et al., 1981) that octopine type plasmids pTiB6S3 and pTiAch5 are identical. Furthermore, no difference was observed between the restriction patterns of both plasmids for enzymes Sma I, Hpa I, Kpn I and Xba I(De Vos et al., 1981).

In order to determine the degree of homology between octopine and nopaline Ti plasmids, the octopine plasmid pTiAch5 was hybridised to the nopaline plasmid pTiC58 using Southern blot hybridisation and

electron microscope heteroduplex analysis. For the Southern blot hybridisations, total labelled pTiAch5 DNA was hybridised to Southern blots of restriction fragments from a series of hybrid plasmids containing overlapping segments of the whole pTiC58 plasmid(Engler et al., 1981). Reciprocal experiments were also carried out. It was found that the common sequences between the two plasmids was approximately 30% and these regions were restricted to four major stretches of homology, named A, B, C and D (Figure 1.3). Using electron microscope analysis, it was found that two regions of the two plasmids were distributed in the same relative order as compared to a common reference point and two were inverted. Three regions contained a number of small, asymmetrical loops. Several regions distributed over the common DNA sequences were found to be only partially homologous. Several of the homologous and non homologous regions were correlated with different functions mapped on the plasmids by insertion and deletion mutagenesis(Schell et al., 1979; Holsters et al., 1980; De Greve et al., 1981). The A region of homology was found to overlap the T-DNA and was directly involved in the determination of oncogenicity. The right hand end of the B region was found to encode replication and incompatibility functions. Exclusion of bacteriophage AP1 and oncogenicity functions were located in the C region.

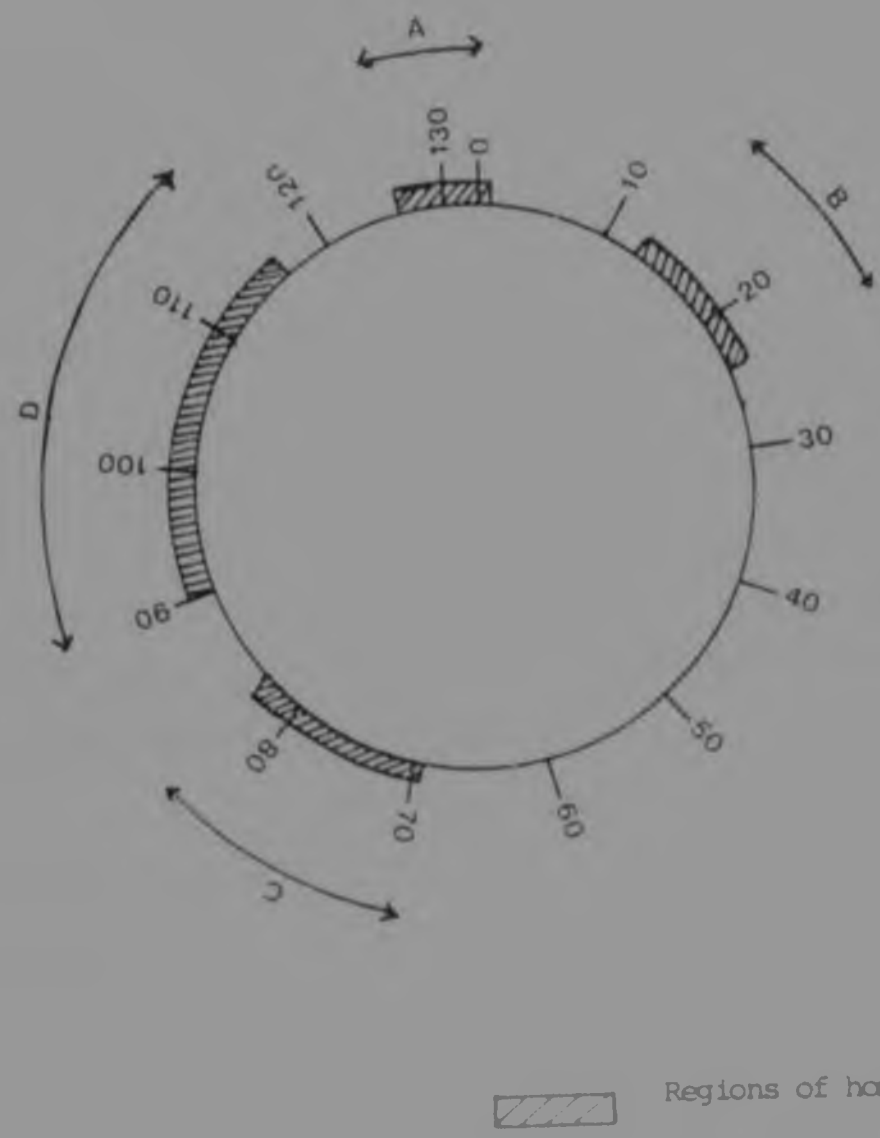


Figure 1.3. Localisation of homologous and non-homologous regions on the pTiC58 restriction map(from Engler et al., 1981) The units of the map are given in megadaltons.

Homologous region D was found to be involved indirectly in the Ti plasmid capacity for plant cell transformation(Engler *et al*., 1981).

It has been established(Panagopoulos and Psallidas, 1973; Kerr and Panagopoulos, 1977) that all pathogenic strains of *A. tumefaciens* can be divided into three distinct biotypes. Agrobacteria belonging to biotype 1 or 2(Kersters *et al*., 1973), have a broad host range, inducing tumors on most dicotyledonous plants. Isolates belonging to biotype 3 usually have a limited host range; most of them induce tumors on grapevines(Panagopoulos *et al*., 1978; Perry and Kado, 1982). Agrobacteria usually exhibit some degree of host specificity(Anderson and Moore, 1979. It was shown(Loper and Kado, 1979; Thomashow *et al*., 1980b)that the host range of *A. tumefaciens* is determined by the Ti plasmid. Conjugal transfer of a broad host range Ti plasmid to a limited host range strain which induced tumors only on grapevine (*Vitis sp*) elicited the donor phenotype (wide host range) in the transconjugants. Thomashow *et al*.(1980b), utilising purified plasmid DNA from *Agrobacterium* strains isolated from grapevines and shown to have a limited host range(Panagopoulos and Psallidas, 1973; Panagopoulos *et al*., 1978), transformed a strain which had been cured of its Ti plasmid. Transformants showed a virulence response

identical to that of the donor strain with all plants tested. Using restriction enzyme analysis, the transformants were shown to harbour donor strain plasmids(Thomashow *et al*., 1980b).

It is well established that bacteria can defend themselves against closely related strains by producing bacteriocins. In most of the cases studied these toxic compounds are proteins and their action is limited to strains with a receptor for the bacteriocin. The strain producing the bacteriocin is itself immune to the effects of the toxin, usually due to the concomitant production of an immunity protein(Reeves, 1965; Nomura, 1967).

Bacteriocinogenic properties have been observed for agrocin 84(Kerr and Htay, 1974; Tate *et al*., 1979; Kerr, 1980). Agrocin 84 is a nucleotide bacteriocin which is produced by *A. radiobacter* strain K84(Tate *et al*.,1979; Kerr, 1980). It has been characterised as a disubstituted fraudulent nucleotide(Roberts *et al*., 1977; Tate *et al*., 1979) with a 9-(3'-deoxy-α-D-threo-pentafuranosyl)adenine nucleoside core. K84 is an avirulent biotype 2 bacterium which carries a plasmid, pAt84a(30 x 10^6 D) that codes for agrocin production and a larger plasmid, pAt84b(124 x 10^6 D) that codes for nopaline utilization and plasmid

transfer(Ellis and Kerr, 1978; Ellis *et al*., 1979; Ellis and Kerr, 1979).

A close association between the pathogenicity of a strain, nopaline utilization and agrocin 84 sensitivity has been found(Engler *et al*., 1975; Kerr and Roberts, 1976). Strains cured of the nopaline Ti plasmid are resistant to agrocin 84(Sule, 1978) and sensitivity to agrocin has been mapped at 87 Md on the nopaline plasmid pTiC58(Holsters *et al*., 1980).

Since 1972, strain K84 has been used worldwide on thousands of plants for biological control(Moore and Warren, 1979). Susceptible plants are inoculated with the nonpathogenic strain K84 before being grown in soil that may contain pathogenic strains(Kerr, 1980). The mechanism of control is through production of a bacteriocin by the controlling organism(Kerr and Htay, 1974; Kerr, 1980). In Australia and New Zealand, strain K84 has been used successfully to control crown gall in many plants(Kerr, 1969b; Kerr, 1972; New and Kerr, 1972; Kerr, 1974; Htay and Kerr, 1974; Kerr and Panagopoulos, 1977; Moore, 1977; Garrett, 1979; Kerr, 1980; Spiers, 1980). However, in some countries, strain 84 failed to prevent crown gall on some plants(Moore and Warren, 1979). Agrocin 84 was ineffective against the strains causing crown gall on

grapevines in Greece; it also failed to protect apple seedlings in Washington(Moore and Warren, 1979). One of the reasons for the success of biological control in Australia is that the population of A. tumefaciens is fairly homogeneous throughout the country(Kerr, 1974). Most are biotype 2(Keane, Kerr and New, 1970; New and Kerr, 1971), utilize nopaline and are sensitive to agrocin 84(Kerr and Roberts, 1976). More heterogeneity is found elsewhere in the world, for example, in Greece(Kerr and Panagopoulos, 1977; Sule, 1978; Moore and Warren, 1979). Only those biotype 1 and 2 strains sensitive to agrocin 84 were found to be subject to biological control(Kerr and Htay, 1974). Biotype 1 and 2 strains which harbour an octopine Ti plasmid were insensitive to agrocin 84, and were not subject to biological control. Nor were the biotype 3 strains which cause crown gall on grapevines(Kerr and Panagopoulos, 1977). There is therefore a clear need to extend biological control of these strains.

The aim of this study was twofold. The first was to isolate new agrocin producing strains with a broader host spectrum than strain K84 which could be effective in the biological control of crown gall in South Africa. Studies on the genetic basis for the production of agrocin by A. tumefaciens strain D286, and its mode of action, were carried out. The host

range of strain D286 was determined and sensitivity to it mapped on the nopaline Ti plasmid. The second aim was to transfer the agrocin 84 encoding plasmid of strain 396::Tn7 to a benign soil species, namely, *Rhizobium*.

2. DETECTION OF AGROCIN PRODUCTION AND/OR SENSITIVITY TO AGROCIN IN LOCAL ISOLATES OF *AGROBACTERIUM TUMEFACIENS*.

2.1 SUMMARY

Eighteen local isolates of *A. tumefaciens* were screened for the production of and/or sensitivity to an agrocin. Six (30%) of the local strains produced an agrocin. One strain, D286, produced an agrocin with a broader host range than agrocin 84. Eight (32%) of the strains tested (including strains obtained from M. van Montagu, Ghent) were sensitive to D286 agrocin. The agrocin produced by strain D286 was active against strains harbouring the nopaline, octopine and agropine type Ti plasmids. Only strains carrying a Ti plasmid appeared to be sensitive to the agrocin; strains lacking a Ti plasmid were resistant.

2.2 INTRODUCTION

Successful biological control of crown gall with *A. radiobacter* strain K84 which produces an agrocin, 84, has been reported in southern Australia and in several other countries(Kerr, 1972; New and Kerr, 1972; Htay and Kerr, 1974; Kerr and Panagopoulos, 1977; Moore, 1977; Moore and Warren, 1979; Kerr,

1980). This represents the first commercial use of a specific microorganism to control a plant pathogen in soil and it is the first commercial use of a bacterium to control any plant disease(Kerr, 1980). The ratio of pathogen to non-pathogen is extremely important to prevent the formation of pathogenic strains which may lead to the breakdown of biological control. A ratio of one or less than one is essential. However, some strains of A. tumefaciens are insensitive to strain K84 in vitro(Kerr and Htay, 1974; Kerr and Panagopoulos, 1977; Moore and Warren, 1979), and in some cases strain 84 has been shown not to prevent tumor production by these pathogens on susceptible hosts(Kerr and Panagopoulos, 1977). Kerr and Htay(1974) noted the presence of pathogens insensitive to agrocin 84, and speculated that it should be possible to isolate further bacteriocin producing strains capable of inhibiting such pathogens. Cases have been reported where agrocin producing strains inhibit growth of another Agrobacterium strain in vitro but are ineffective in the field(Cooksey and Moore, 1980). It was proposed that the failure to control sensitive pathogenic strains may have been the result of a lack of bacteriocin production by the antagonists in vivo(Cooksey and Moore, 1980), or poor growth of the antagonists at the wound site(Ellis and Kerr, 1978). On some hosts, A. radiobacter strain 84 has been

shown to control pathogenic strains that were insensitive to agrocin 84 *in vitro* (Moore, 1977). This may suggest that other mechanisms, such as competition for infection sites are utilized by strain 84 to prevent infection of some host species (Lippincott and Lippincott, 1969).

In a study on the effect of strain 84 on pathogenic European strains, it was found that strain 84 failed to control 7 out of 14 strains (Moore and Warren, 1979). It also failed to protect field grown apple seedlings in Washington and rose plants in Texas and Pennsylvania. In Greece, no biotype 3 strain (isolated from grapevines) was subject to biological control by strain 84 (Kerr and Panagopoulos, 1977). In contrast, crown gall has been reduced to less than 1% on commercially produced roses in Australia and New Zealand (Moore and Warren, 1979). It was therefore important to look for new antagonists for strain 84 insensitive pathogens.

Deep and Young (1965) showed that fungicide treatments prior to planting increased the incidence of crown gall in cherry seedlings, suggesting the presence of natural fungal competitors. Cooksey and Moore (1980) tried to isolate fungal and bacterial antagonists for biological control of crown gall, particularly for control of *A. tumefaciens* strains not subject to

control by strain 84. They found that certain strains of Penicillium, Aspergillus, Bacillus, Pseudomonas and A. radiobacter reduced the incidence of crown gall on mazzard cherry seedlings. Some of the antagonists reduced the incidence of galling, depending on the group of pathogens used, but none was as effective as strain 84. However, the trials showed that there are microorganisms other than Agrobacteria that could influence crown gall disease.

Having postulated that bacteriocins could exist, which are able to inhibit agrocin 84 resistant strains(Kerr and Htay, 1974), Kerr and Panagopoulos(1977) have described the isolation of new agrocin producing strains from soil. Only one soil sample yielded bacteriocinogenic isolates. However, these isolates did not appear to have potential for control of crown gall in Greece since they did not control agrocin 84 resistant strains.

In view of the fact that not all local strains of A. tumefaciens found in South Africa are sensitive to the agrocin produced by strain 84, local isolates of A. tumefaciens were screened for the production of new agrocins, which may be used to extend biological control of crown gall in South Africa. Our approach was different from that of Kerr and Panagopoulos(1977) who isolated A. tumefaciens

strains from soil. We have tested isolates which were located around gall areas on plants for the production of agrocins.

2.3 MATERIALS AND METHODS

2.3.1 Media are listed in the appendix

2.3.2 Agrocin production and sensitivity tests

Two methods were used to detect the production of an agrocin or sensitivity to an agrocin:

(a) The method of Kerr and Htay(1974) was used. A loopful of the agrocin producing strain was applied as a 5 mm square to the centre of a minimal medium A plate containing 0.2% sodium glutamate or 0.02% glucose. The plates were incubated at 26°C for 48 hours. After incubation the plates were sterilised for 15 min by inversion over a square of filter paper soaked in chloroform. A loopful of the sensitive indicator strain was suspended in 0.5 mls of water. Three millilitres of molten agar(0.6%) containing 0.01 M $MgSO_4$ pH 7.2 was added to the suspension and poured on to the plate. When the soft agar had set, the plates were incubated at 26°C for 48 hrs after which a halo of inhibition of growth could be seen around the agrocin producing strain.

(b) The method of Meyer-Harting *et al.*(1972) was also

used. Overnight cultures of the agrocin producing strain and the agrocin sensitive str were made in LB. Three millilitres of molten agar(0.6%) was added to 0.2 mls of the sensitive indicator strain and poured on to a minimal medium plate. When the soft agar had set, wells were punched in the agar and the agrocin producing strain was added to the well. The plates were incubated at 26°C for 24-48 hrs after which a halo of inhibition was observed around the well.

2.3.3 Bacterial strains

Local strains of A. tumefaciens were tested for the production of and/or sensitivity to an agrocin. These strains, isolated from crown galls on a variety of plants in South Africa, were supplied by the Department of Plant Protection (Table 2.1). Other strains used which are listed in Table 2.2 were supplied by M. van Montagu(Ghent). As A. radiobacter K84 is a very slow growing strain, a pathogenic isolate of A. tumefaciens carrying the agrocin 84 producing plasmid, pAt396, strain 396, was tested. This strain grows faster than K84. However, a Tn7 containing derivative, 396::Tn7 produced higher levels of agrocin 84. Therefore this strain was used. Whether the transposon Tn7 has any connection with the increased levels of agrocin produced

Table 2.1 Origin of South African strains screened for agrocin production and/or sensitivity.

Strain number	Origin
D208	Begonia
D209	Chrysanthemum
D210	Chrysanthemum
D216	Bauhinia
D236	Bauhinia
D259	Yellow Jasmine
D276	Eucalyptus
D284	Eucalyptus
D285	Eucalyptus
D286	Eucalyptus
D287	Eucalyptus
53	Grapevine
65	Peach
71	Peach
72	Unknown
115	Grapevine
158	Grapevine
165	Grapevine

Table 2.2 Bacterial strains obtained from the Rijksuniversiteit,Ghent, used in the screening for agrocins and/or sensitivity to agrocins

Strain number	Ti plasmid	Other plasmids	Origin
C58	pTiC58 (nopaline)	Cryptic	Hamilton and Fall (1971)
C58C1	None	Cryptic	C58 cured of pTiC58
Ach5	pTiAch5 (octopine)	Cryptic	Hamilton and Fall (1971)
Ach5C3	None	Cryptic	Ach5 cured of pTiAch5
K84	None	Cryptic pAt84a pAt84b	Kerr and Htay (1974)
396	pTi396 (agropine)	Cryptic pAt396	M. van Montagu
396::Tn7	pTi396 (agropine)	Cryptic pAt396	Transposon Tn7 mutagenesis of 396, M. van Montagu

is unlikely (see Chapter 6).

2.4 RESULTS

Of the eighteen local isolates of *A. tumefaciens* screened, 6 were found to produce an agrocin which inhibited the growth of at least one of the strains tested (Table 2.3). Strain D286 was found to produce an agrocin with a broader host range than that of strain K84 or 396::Tn7.

2.5 DISCUSSION

Of the 18 local isolates tested 6 were found to produce an agrocin which inhibited the growth of at least one other strain. Strain D286 produced an agrocin with a broader host range than the agrocins of strain K84 and 396::Tn7, however, it also appeared as though sensitivity to the agrocin produced by strain D286 was Ti plasmid encoded. That is, it killed C58 which harbors a nopaline Ti plasmid, but not C58C1 which is a derivative of C58 cured of its Ti plasmid; it killed 396::Tn7 which harbors an agropine Ti plasmid but not strain K84 which lacks a Ti plasmid. D286 agrocin inhibited the growth of a local strain carrying an

Table 2.3 Agrocin production and sensitivity of local isolates of *A. tumefaciens*[1]

strain tested for agrocin sensitivity	strain tested for agrocin production												
	53	65	71	72	115	158	165	D208	D209	D210	D216	D236	D259
53	R	R	R	R	R	R	R	R	R	R	R	R	R
65	R	R	R	R	R	R	R	R	R	R	R	S	S
71	R	R	R	R	R	R	R	R	R	R	R	R	R
72	R	R	R	R	R	R	R	R	R	R	R	R	R
115	R	R	R	R	R	R	R	R	R	R	R	R	R
158	R	R	R	R	R	R	R	R	R	R	R	R	R
165	R	R	R	R	R	R	R	R	R	R	R	R	R
D208	R	R	R	R	R	R	R	R	R	R	R	R	R
D209	R	R	R	R	R	R	R	R	R	S	R	R	R
D210	R	R	R	R	R	R	R	R	R	R	R	S	S

[1]Strains were tested against each other for the production of an agrocin or sensitivity to an agrocin. The methods of Mayr-Harting *et al.*,(1972) and Kerr and Htay(1974) were used.

R = resistant, S = sensitive.

Table 2.3 cont.'d

	strain tested for agrocin production												
	53	65	71	72	115	158	165	D208	D209	D210	D216	D236	D259
Strain tested for agrocin sensitivity													
D216	R	R	R	R	R	R	R	R	R	R	R	R	R
D236	R	R	R	R	R	R	R	R	R	R	R	R	R
D259	R	R	R	R	R	R	R	R	R	R	R	R	R
D276	R	R	R	R	R	R	R	R	R	R	R	R	R
D284	R	R	R	R	R	R	R	R	R	R	R	R	R
D285	R	R	R	R	R	R	R	R	R	R	R	R	R
D286	R	R	R	R	R	R	R	R	R	R	R	R	R
D287	R	R	R	R	R	R	R	R	R	R	R	R	R
K84	R	R	R	R	R	R	R	R	R	R	R	R	R
396	R	R	R	R	R	R	R	R	R	R	R	R	R
396::Tn7	R	R	R	R	R	R	R	R	R	R	R	R	R
C58	R	R	R	R	R	R	R	R	R	R	R	R	R
C58C1	R	R	R	R	R	R	R	R	R	R	R	R	R
Ach5	R	R	R	R	R	R	R	R	R	R	R	R	R
Ach5C3	R	R	R	R	R	R	R	R	R	R	R	R	R

Table 2.3 cont.'d

	strain tested for agrocin production											
	D276	D284	D285	D286	D287	K84	396	396::Tn7	C58	C58C1	Ach5	Ach5C3
Strain tested for agrocin sensitivity												
53	R	R	R	R	R	R	R	R	R	R	R	R
65	S	R	R	S	R	S	S	S	R	R	R	R
71	R	R	R	R	R	R	R	R	R	R	R	R
72	R	R	R	S	R	R	R	R	R	R	R	R
115	R	R	R	R	R	R	R	R	R	R	R	R
158	R	R	R	R	R	R	R	R	R	R	R	R
165	R	R	R	R	R	R	R	R	R	R	R	R
D208	R	R	R	S	R	R	R	R	R	R	R	R
D209	R	R	S	R	R	R	R	R	R	R	R	R
D210	S	R	R	R	R	R	R	R	R	R	R	R

Table 2.3 cont.'d

Strain tested for agrocin sensitivity	strain tested for agrocin production											
	D276	D284	D285	D286	D287	K84	396	396::Tn7	C58	C58C1	Ach5	Ach5C3
D216	R	R	R	R	R	R	R	R	R	R	R	R
D236	R	R	R	R	R	R	R	R	R	R	R	R
D259	R	R	R	R	R	R	R	R	R	R	R	R
D276	R	R	R	S	R	R	R	R	R	R	R	R
D284	R	R	R	R	R	R	R	R	R	R	R	R
D285	R	R	R	R	R	R	R	R	R	R	R	R
D286	R	R	R	R	R	R	R	R	R	R	R	R
D287	R	R	R	R	R	R	R	R	R	R	R	R
K84	R	R	R	R	R	R	R	R	R	R	R	R
396	R	R	R	S	R	R	R	R	R	R	R	R
396::Tn7	R	R	R	S	R	R	R	R	R	R	R	R
C58	R	R	R	S	R	S	S	S	R	R	R	R
C58C1	R	R	R	R	R	R	R	R	R	R	R	R
Ach5	R	R	R	S	R	R	R	R	R	R	R	R
Ach5C3	R	R	R	R	R	R	R	R	R	R	R	R

agropine Ti plasmid, strain D208, and a strain which carries an octopine Ti plasmid, strain Ach5, but not Ach5C3 its cured derivative.

Strain D286, the agrocin producer, was selected for further study due to the fact that it inactivated four of the local strains tested as well as strains 396, 396::Tn7, C58 and Ach5. A local isolate, D208, was used as the sensitive indicator strain.

3. PRODUCTION OF AGROCIN BY *A. TUMEFACIENS* STRAIN D286.

3.1 ABSTRACT

Production of agrocin by D286 was studied. The agrocin was found to be present in the culture in late log phase. The agrocin was produced in Luria broth between OD_{540} 1.26 and 1.36 with a peak of activity at OD_{540} 1.36, after which agrocin activity rapidly disappeared. The agrocin was only found extracellularly, and not cell bound or intracellularly. The agrocin produced in late log phase was unstable in the presence of growing D286 cells. However, if the cells were removed from the agrocin, the agrocin was stable. The agrocin degrading or destabilising substance was not located or identified in any fraction of a cell culture, viz., the intracellular, cell bound or extracellular fractions. There was a marked increase in production of agrocin on minimal medium plates containing sodium glutamate compared to glucose minimal medium. However, this difference in agrocin production was not observed in liquid medium. Production of agrocin in liquid minimal medium containing sodium glutamate or glucose followed a similar pattern as in LB with a peak of agrocin activity at OD_{540} 0.175 and 0.325,

respectively. The same level of agrocin was produced in both media but this occurred earlier in the growth phase in sodium glutamate containing medium.

Treatment of strain D286 with ultraviolet light and mitomycin C, agents which have been shown to induce higher levels of bacteriocin production, did not induce agrocin production. Strain D286 was screened for the presence of plasmids. Two plasmid bands were observed. It is not known whether one of these bands represents a plasmid which encodes agrocin production. Coumermycin, ethidium bromide, flavine, acridine orange and mitomycin C did not 86 of agrocin production.

The formation of crown gall tissue on potato slices by virulent strains C58, D208 and Ach5 was inhibited by agrocin D286. Heat killed D286 cells did not inhibit gall formation by C58 and D208, indicating that agrocin production by strain D286 and not cell competition for infection sites was responsible for the control of gall formation.

Of 16 Rhizobium strains tested, only R. meliloti strain RF14 and XHH1, a strain belonging to the cowpea miscellany were found to be sensitive to D286 agrocin. When comparing the effectiveness of strains D286 and K84, it was found that agrocin D286 was active

against a larger number of A. rhizogenes strains than agrocin 84.

3.2 INTRODUCTION

The study of bacteriocins began in 1925 when Gratia (see Mayr-Harting et al., 1972) discovered a highly specific antibiotic produced by one strain of Escherichia coli which was active against another strain of the same species. Since then, bacteriocins, especially the colicins have been extensively studied. It has been found that bacteriocinogenic strains only produce the bacteriocin at certain stages of development of the cell culture, and only under certa .. conditions of growth(Reeves, 1965). The factors controlling the synthesis of certain bacteriocins are incompletely understood.

The conditions for production of bacteriocins appear to vary from one strain to another. The time of incubation is important for maximum bacteriocin titres to be obtained,Mayr-Harting et al., 1972). For example, the bacteriocin streptocin STH is maximally produced during the exponential growth phase, with a sharp decline in the level of the bacteriocin just before the culture enters stationary

phase. Another bacteriocin, streptococcin A-FF22 is produced late in the exponential phase, and activity slowly decreases on prolonged incubation(Mayr-Harting *et al.*, 1972). Production of agrocin 84 by *A. radiobacter* strain K84 is directly related to growth of the bacteria. Bacteriocin production occurs simultaneously with bacterium growth, that is, it increases in the exponential phase of bacterial growth and declines as growth enters the stationary phase, and then remains static(Spiers,1980). pH, temperature and nutritional factors may have profound effects on the production of the bacteriocin. Agrocin 84 is only produced between 25° and 28°C. It has been shown that the presence of glucose in the culture medium can inactivate colicin K or inhibit its synthesis(Mayr-Harting *et al.*, 1972). It is therefore logical to state that optimum conditions for the production of bacteriocins are those which allow optimum expression of the bacteriocinogenic factors.

Jacob *et al.* (see Mayr-Harting *et al.*, 1972) first showed that biosynthesis of a particular colicin was inducible by ultraviolet light. They emphasized the analogy between colicinogeny and lysogeny. Induction of bacteriocin production in several bacteriocinogenic strains was subsequently achieved by a variety of means known to be effective for

induction of temperate phages. The process of induction is of great practical importance because of the increased bacteriocin yield which can be obtained. The bacteriocin may be harvested after only a few hours as opposed to a day or more in an uninduced culture. Ultraviolet light is a powerful mutagen; it induces the formation of pyrimidine dimers in the DNA molecule. Different bacteria are sensitive to different doses of ultraviolet light. I is therefore important to determine the optimal conditions for each system.

The most commonly used chemical inducer of bacteriocin production is mitomycin C. Mitomycin C is known to inhibit DNA synthesis as well as to form cross linkages in the DNA molecule(Herskowitz, 1977).

Acridine orange, acriflavine, ethidium bromide, coumermycin, and mitomycin C have been used to cure Col and F factors(Mayr-Harting et al., 1972; Miller, 1974; Danilevskaya and Gragerov, 1980). Acridines cause mutations in the DNA either by binding to the outside of the molecule or by inserting themselves between adjacent nucleotides(Herskowitz, 1977). Mutagenesis by acridines may involve the chromosomal replication mechanism and/or the mechanism for the repair of the mutation. Kado(1976) observed that the tumor inducing ability was lost in the fraction of an

A. tumefaciens population surviving treatment with acridine dyes and ethidium bromide. Low concentrations of the antibiotic coumermycin A1 have been shown to inhibit the supercoiling activity of DNA gyrase by binding itself to the enzyme's B subunit. Coumermycin effectively eliminated plasmids pBR322, pMB9 and other Col E1 related plasmids from *E. coli* K12. The curing action of the antibiotic resulted from inhibition of plasmid replication and plasmid degradation(Danilevskaya and Gragerov, 1980).

Agrobacterium rhizogenes is the causative agent of hairy root, a neoplastic disease of dicotyledonous plants, characterised by root proliferation at the sites of bacterial infection(Costantino *et al.*, 1981). *A. rhizogenes* is taxonomically related to *A. tumefaciens*. Like crown gall, hairy root disease is associated with the presence of a large plasmid(Moore *et al.*, 1979; White and Nester, 1980a), part of which (T-DNA) becomes inserted into the host genome(Chilton *et al.*, 1982). Ri (root inducing) plasmids share extensive homology with each other(Costantino *et al.*, 1981) and with the oncogenicity regions of octopine and nopaline plasmids of *A. tumefaciens*(White and Nester, 1980a; Risuleo *et al.*, 1982).

The genus Agrobacterium is closely related to the genus Rhizobium. Bacteria belonging to both groups are natural inhabitants of the soil. Work on these two groups of organisms reaffirmed the close relationship between them. This relationship has been the basis for their inclusion in the family Rhizobiaceae through several editions of Bergey's manual. The genus Rhizobium has traditionally been divided into two groups(Elkan, 1981). The term "fast growers" commonly designates the rhizobia associated with alfalfa, clover, bean and pea. In culture they grow much faster (less than half the doubling time) than the "slow growers" exemplified by soybean, cowpea and lupine rhizobia. The "fast growers" are more closely related to Agrobacterium than the "slow growers". Many species of Rhizobium are responsible for nitrogen fixation. Therefore if an agrocin producing strain is to be used as a means of biological control of crown gall, it is important to ensure that the agrocin does not inhibit growth of the Rhizobium strains.

Production of agrocin by strain D286 in Luria broth, and minimal medium containing sodium glutamate or glucose was determined. An attempt was made to induce the agrocin so that larger quantities could be obtained for purification studies. To determine whether agrocin production was encoded by a plasmid

in strain D286, plasmid screens and curing studies were carried out. The effect of agrocin D286 on *A. rhizogenes* and *Rhizobium* strains was also determined.

3.3 MATERIALS AND METHODS

3.3.1 Media and chemicals are given in the appendix

3.3.2 Production of agrocin by strain D286 in Luria broth

An overnight culture of strain D286 was diluted 1:30 in LB, and incubated on an orbital shaker at 26°C. Samples of the culture were taken at various time intervals and the cell density measured at A_{540} using an MSE Spectroplus spectrophotometer. The cells were pelleted at 8500 x g at 4°C for 10 mins in a JA20 rotor using a Beckman J21B centrifuge. The supernatant was diluted and two hundred microlitres of each dilution was assayed for agrocin activity on minimal medium A containing sodium glutamate using the well plate method of Mayr-Harting *et al.* (1972). The sensitive indicator strain used was D208. The bacteriocin activity (arbitrary units, AU) was defined as the reciprocal of the highest dilution to give a detectable zone of inhibition.

As a control, production of agrocin by strain

396::Tn7 was determined. C58 was used as the sensitive indicator strain.

3.3.3 Location of the bacteriocin

In order to determine the location of the agrocin, the pellets obtained as described in section 3.3.2 at different time intervals were washed in 1 M NaCl and the supernatant assayed for bacteriocin activity. The sodium chloride washed cells were then resuspended in the same volume (2 mls) of 0.02 M Tris-HCl buffer, pH 8.2 and disrupted by sonication at an amplitude of 8 microns for 2 mins in an MSE sonicator. The temperature was not allowed to rise above 10°C during the process. The disrupted cells were then centrifuged at 25000 x g for 15 mins. The supernatant was assayed for agrocin activity.

3.3.4 Loss of agrocin activity

In order to ascertain whether agrocin degradation could be assigned to cells in a particular growth phase, crude agrocin in LB was mixed on a 1:1 basis with whole cells (washed in 1 M NaCl or unwashed), sonicated cells and cell supernatants. These fractions were obtained from cells grown to an OD_{540} of between 0.72 and 1.69. The agrocin and the individual fractions were incubated for 16 hrs at

26°C. Each sample was assayed for agrocin activity.

3.3.5 Comparison of agrocin production in minimal medium A with glucose or sodium glutamate.

3.3.5.1 Comparison of agrocin production on solid medium

The agrocin producing strains D286, 396::Tn7 and K84 were patched as 5 mm squares on to minimal medium A plates containing either sodium glutamate or glucose. The plates were incubated at 26°C or 28°C for 48 and 72 hrs. After the specified period of incubation, the plates were sterilised by inversion over a square of filter paper containing chloroform. The following strains were seeded as an overlay on individual plates: D208, C58, C58C1, 396::Tn7, K84 and D286.

3.3.5.2 Comparison of agrocin production in liquid medium containing sodium glutamate or glucose

D286 was grown overnight in minimal medium A broth containing sodium glutamate or glucose. The cultures were diluted 1:20 in the appropriate medium. At various times, aliquots were taken, the optical density (A_{600}) read, and the supernatant of the sample culture assayed for agrocin activity as in section 3.3.2.

3.3.6 Induction of the bacteriocin

An overnight culture of D286 was diluted 1:10 until an OD_{540} of 0.2 was reached. Mitomycin C to final concentrations 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, and 2.0 µg/ml was added to 10 ml samples of the culture. The cultures were incubated on a roller drum for 3 hrs at 26°C. After incubation, the viable count of the cultures was determined. The cells were washed, resuspended in fresh LB, and reincubated at 26°C. The agrocin was assayed after 0, 24, 26, 28, 30, and 48 hrs incubation

Induction by ultraviolet light was determined by exposing 5 ml samples of an exponentially growing culture of D286, OD_{540} 0.28, (previously resuspended in 0.8% NaCl) to a Hanau ultraviolet lamp (0,6 Jm^{-2} sec^{-1}) for 0, 15, 30, 60, 90, and 120 secs. After irradiation, the cultures were centrifuged and the pellets resuspended in LB. The culture was incubated at 26°C for 0, 24, 26, 28, 30, and 48 hrs at which times the agrocin was assayed. Viable count of the irradiated cells were also determined.

3.3.6 Induction of the bacteriocin

An overnight culture of D286 was diluted 1:10 until an OD_{540} of 0.2 was reached. Mitomycin C to final concentrations 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, and 2.0 µg/ml was added to 10 ml samples of the culture. The cultures were incubated on a roller drum for 3 hrs at 26°C. After incubation, the viable count of the cultures was determined. The cells were washed, resuspended in fresh LB, and reincubated at 26°C. The agrocin was assayed after 0, 24, 26, 28, 30, and 48 hrs incubation

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3.3.7 Curing of agrocin production in strains D286 and 396::Tn7, and of the Ti plasmid in C58.

Strains D286, 396::Tn7, C58 and C58C1 (control which does not carry a Ti plasmid) were plated on to LA plates containing various concentrations of curing agents, viz., ethidium bromide (250, 500 and 750 µg/ml), acriflavine (75, 100, 125, 150, 650 µg/ml), coumermycin (0.07, 0.1, 0.15 and 0.2 µg/ml), mitomycin C (0.5, 1.0, 1.5 and 2,0 µg/ml) and acridine orange (500 and 1000 µg/ml). The plates were incubated at 26°C for 72 hrs. One hundred colonies from each concentration of each curing agent were purified on LA. D286 and 396::Tn7 were tested for the loss of agrocin production using indicator strains D208 and C58 respectively. C58 was tested for the loss of the Ti plasmid, that is, by the loss of sensitivity to the agrocin produced by 396::Tn7.

3.3.8 Screening for plasmids in A. tumefaciens strains

The method of Kado and Liu(1981) was used to screen for plasmids in A. tumefaciens. Cells were grown overnight at 26°C in LB. The cells were pelleted by centrifugation in a Beckman Centrifuge model J21B in a JA20 rotor at 5700 x g and the pellet resuspended in 1 ml E buffer. The cells were lysed by the

addition of 2 ml of lysing solution (3% SDS in 50 mM Tris pH 12.6) which was mixed by br . agitation. The solution was heated at 95°C for various lengths of time in a waterbath. After heat treatment the solution was immediately placed on ice. Two volumes of phenol:chloroform (1:1, vol/vol) were added. The solution was emulsified by brief shaking. The solution was centrifuged at 5700 x g for 15 min at 4°C in a JA20 rotor. The upper aqueous phase was transferred to an Eppendorf tube using a glass Pasteur pipette, avoiding the precipitate at the interfac

A 50µl sample of the plasmid was mixed with 5µl of tracking dye on a piece of parafilm. To minimize shearing of the plasmids, samples were withdrawn with a 50µl Dade micropipette.

Agarose gel electrophoresis was performed using a 0.7% agarose gel (Seakem). The buffer used was E buffer, pH 7.9. Electrophoresis was carried out at 100 V for approximately 2.5 hrs until the tracking dye had migrated approximately 8 cm. The gel was stained with 0.5 µg/ml of ethidium bromide for 30 min. The gels were destained in distilled water for 45 mins. The gel was illuminated from below with a Chroma-Vue transilluminator, model C-63, to visualise the DNA bands. Photographs were taken with a

Polaroid CU-5 land camera, with a type 665 film. Negatives were scanned at 520 nm using the negative scanning device of a Varian Cary 210 spectrophotometer.

3.3.9 Sensitivity of A. rhizogenes and Rhizobium strains to agrocin D286

The effect of agrocin D286 on A. rhizogenes (Table 3.2) and Rhizobium strains (Table 3.1) was determined.

3.3.10 Laboratory tests to determine the effectiveness of agrocin D286 in the control of crown gall formation

Tests were carried out on Bintje potato slices. Potatoes were surface sterilised in 1% sodium hypochlorite for 30 mins. The core of the potato was cut out, sliced, and placed on to 0.8% agar plates containing 50 units of nystatin per millilitre. Overnight cultures of virulent (C58, Ach5, D208), avirulent (C58C1 and Ach5C3), and agrocin producing (D286 and K84) bacteria were inoculated on to the potato slices. Virulent and agrocin producing bacteria were mixed in different ratios to determine the effect of lower D286 cell numbers in controlling tumor formation. Heat killed D286 cells were mixed

Polaroid CU-5 land camera, with a type 665 film. Negatives were scanned at 5[illegible]0 nm using the negative scanning device of a Varia[illegible] Cary 210 spectrophotometer.

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Table 3.1 Rhizobium strains and their origin

Rhizobium strain number	Plant species on which the are effective and from which they were isolated.
Rhizobia from the cowpea miscellany	
XSL1	Arachis hypogaea (groundnut) and Vigna unguiculata (cowpea
XBL6	Desmodium intortum
XBQ5	Desmodium uncinatum
XCR7	Leucaena leucocephala
XCT9	Lotononis bainsii
XCV14	Lotus corniculatus and L. tenuis
XIH1	Onobrychis viciafolia
XIC1	Stylosanthes fulanensis
XNT1	Lotus pedunculatus
XIX1	Desmodium cunnata
Rhizobium meliloti	
RF14	Medicago sativa (lucerne)
Rhizobium trifolii	
SR4	Trifolium spp.
Rhizobium leguminosarum	
[illegible]	Pisum sativum (peas) and Vicia spp. (vetches)
Rhizobium phaseoli	
UD2	Phaseolus spp. (beans)
Rhizobium lupini	
VK10	Lupinus and ornithopus species
Rhizobium japonicum	
[illegible]	Glycine max (soybeans)

Table 3.2 A. rhizogenes strains which were tested for sensitivity to agrocins D286 and 84. Strains were obtained from the Rijksuniversiteit, Gent

Strain number	Reference
15834	Costantino et al., 1981
Tr1	
Tr8.3	
Tr101	Costantino et al., 1981
Tr107	
ATCC 11325	White and Nester, 1980b

Table 3.2 *A. rhizogenes* strains which were tested for sensitivity to agrocins D286 and 84. Strains were obtained from the Rijksuniversiteit, Ghent

Strain number	Reference
15834	Costantino *et al.*, 1981
Tr1	
Tr8.3	
Tr101	Costantino *et al.*, 1981
Tr107	
ATCC 11325	White and Nester, 1980b

with C58 and D208 cells in equal numbers before inoculation on to slices to determine whether competition for infection sites or agrocin production was responsible for control of gall formation. Plates were sealed with parafilm to retain moisture, and incubated at 26°C for approximately 14 days.

3.4 RESULTS

3.4.1 Production of agrocin by D286 in Luria broth

Production of agrocin by D286 in Luria broth is shown in Figure 3.1. Production of agrocin by 396::Tn7 is also shown.

3.4.2 Location of the agrocin

All the agrocin activity was found to be extracellular. No agrocin was found in the NaCl solution used to wash the cells, indicating that no agrocin was bound to the cell surface. Similarly, no further activity was found in the disrupted cells.

3.4.3 Detection of the agrocin degrading substance

Since the agrocin activity disappeared after the peak of activity(OD_{540} 1.36, Figure 3.1), different cell fractions of a D286 culture were assayed for a possible agrocin degrading substance. No agrocin degrading activity was found in any of the fractions tested, as indicated in Table 3.3.

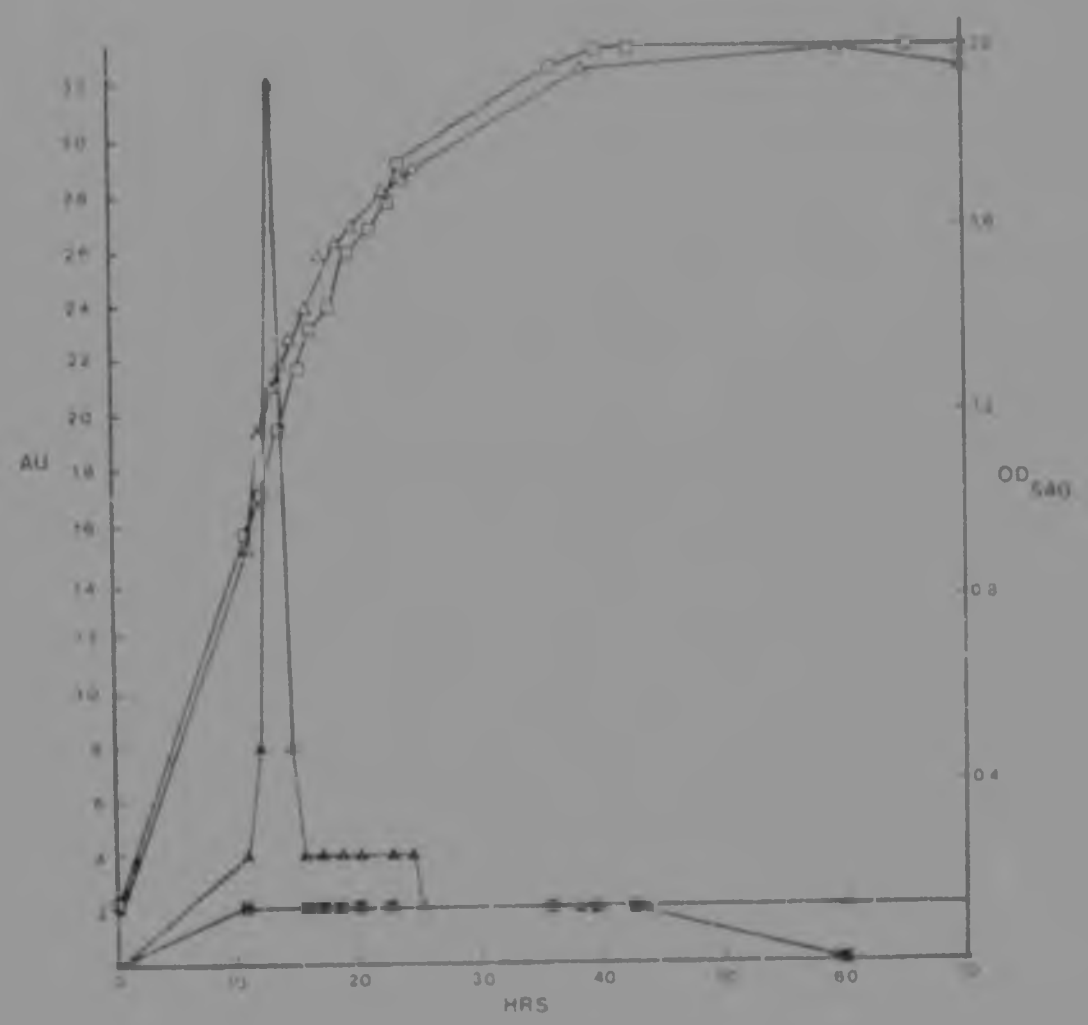


Figure 3.1 Comparison of the growth curve (△—△) and the production of agrocin by D2 (▲—▲) with the growth curve (□—□) and the production of agrocin (■—■) by 396::Tn7. Samples of each culture were taken at different times, the optical density (540) read, and the supernatant of each sample assayed for the amount of agrocin activity. Agrocin activity is expressed as arbitrary units, the reciprocal of the highest dilution which gave positive agrocin activity.

Table 7.1 Effect of fractions of the cell culture on agrocin activity.

Turbidity of the cell culture (OD_{540})	Fraction of the culture added to the agrocin					
	supernatant	unwashed cells	washed cells	wash (NaCl)	sonicate of washed cells	sonicate of washed cells
0.72	+	+	+	+	+	+
0.78	+	+	+	+	+	+
1.08	+	+	+	+	+	+
1.34	+	+	+	+	+	+
1.55	+	+	+	+	+	+
1.6	+	+	+	+	+	+
1.69	+	+	+	+	+	+

+: active agrocin

3.4.4 Comparison of agrocin production in the presence of glucose or sodium glutamate

3.4.4.1 Production of agrocin on solid medium

More agrocin was produced on sodium glutamate than on glucose plates as indicated in Figures 3.2, 3.3 and 3.4. This applied to agrocin D286 as well as agrocin 84 produced by K84 and 396::Tn7. More agrocin was produced over a longer period of time as can be seen in Figure 3.2 when comparing the halos of inhibition produced after 48 and 72 hrs and production of agrocin was slightly enhanced at 28°C as compared to 25°C. A larger halo of inhibition of growth was observed around strain D286 when D208 was used as the sensitive strain (Figure 3.3) than when C58 was used as the sensitive strain (Figure 3.4).

3.4.4.2 Production of agrocin in liquid medium

The growth curve of D286 in MM + sodium glutamate and MM + glucose and the production of agrocin in these media are shown in Figure 3.5. The same pattern of agrocin production was found as in LB. Agrocin was produced earlier in the growth phase in sodium glutamate containing medium than in glucose medium.

Figure 3.2 Comparison of the production of agrocin by D286,396:Tn7 and k84 on minimal medium containing sodium glutamate or glucose at 25°C or 28°C for 48 and 72hrs. The agrocin producing strains were patched on the plates and grown at the appropriate temperature. Agrocin sensitive strains were seeded on to the plates as an overlay. The diameters of the zones of inhibition of growth(mm) of the agrocin sensitive strains D208, C58,396:Tn7 and C58C1 are illustrated here. 'Empty bars' represent the diameter(mm) of the zone of inhibition observed on MM + sodium glutamate plates. Cross hatched bars represent the diameter(mm) of the zone of inhibition observed on MM + glucose.

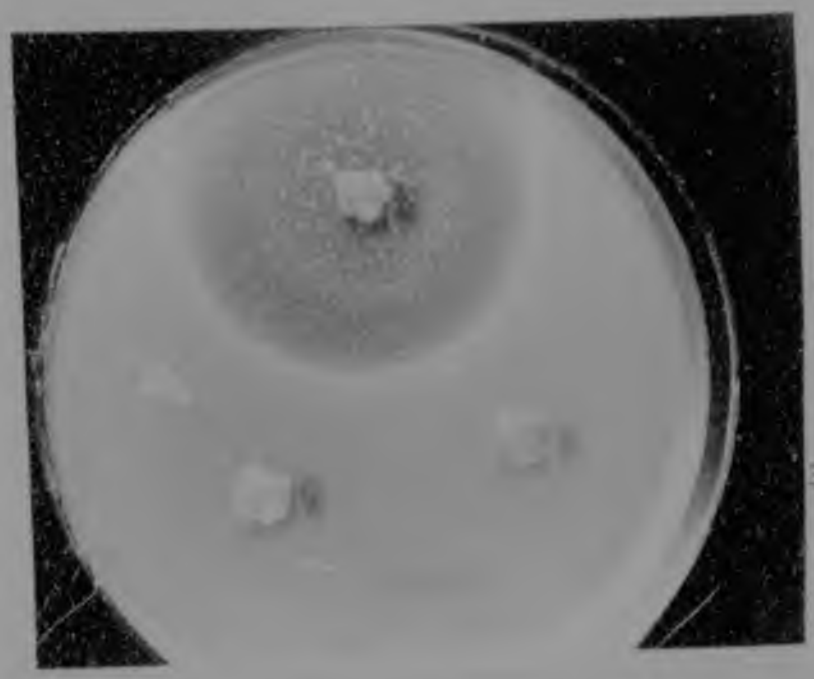


Figure 3.3 Production of agrocin by D286 on MM + sodium glutamate (a) and glucose(b). 396::Tn7, K84 and D286 were patched on to the plates. Plates were indubated at 26°C for 72 hrs. D208 was seeded on to the plates using 0.6% agar. Only D286 produced agrocin to which D208 was sensitive. A smaller zone of inhibition is seen on MM + glucose.

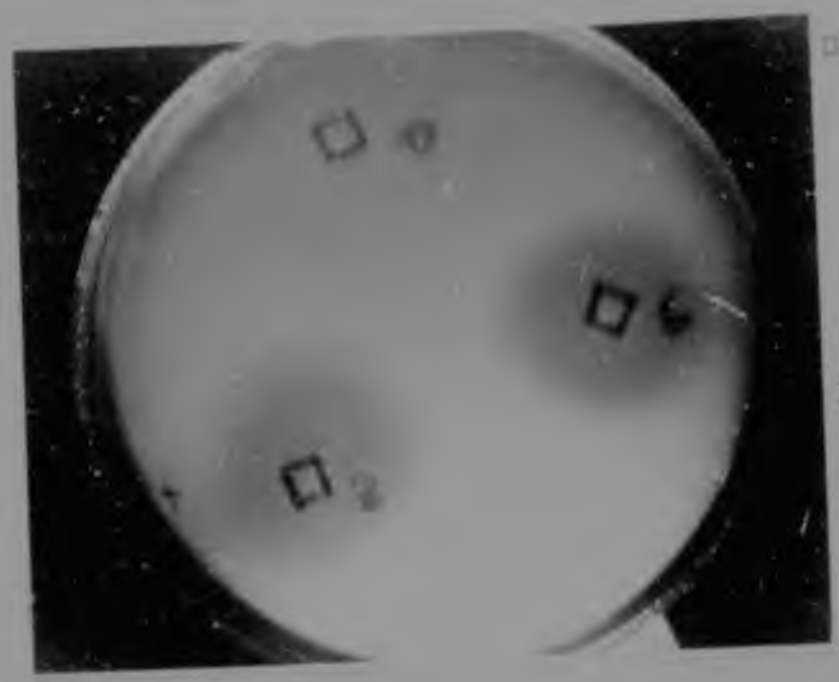
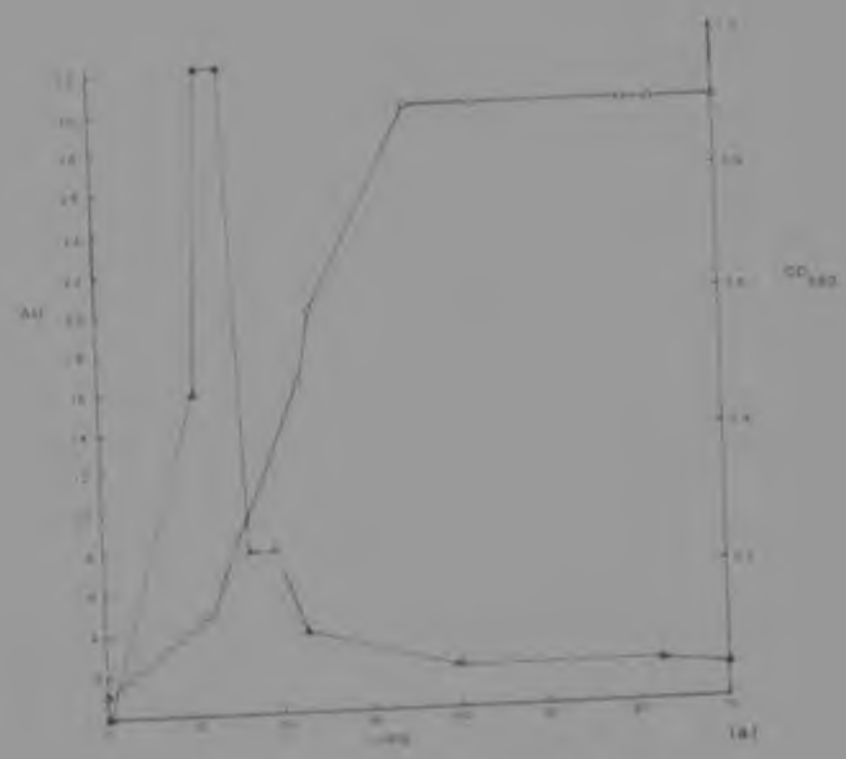
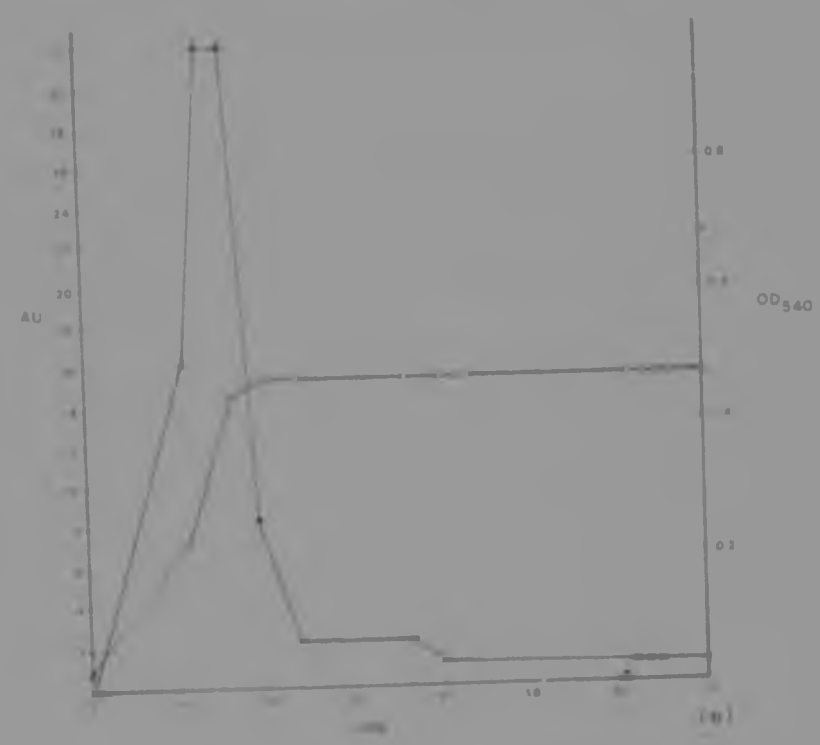


Figure 3.4 Production of agrocin by D286, K84 and 396::Tn7. The three strains were patched on to MM + glucose (a) and MM + sodium glutamate (b) plates. Plates were incubated at 26°C for 72 hrs. CTA was seeded on to the plates using 0.6% agar. A zone of inhibition is seen around all three agrocin producing strains on sodium glutamate containing plates. No zone of inhibition is seen around D286 on glucose plates.





F[illegible] (a–a) [illegible] pr[illegible] [illegible] Figure 3.5(b). [illegible]wth of D266 (a–a) [illegible] of [illegible] [illegible] f the culture were taken at different times. The optical density 54[illegible] read, and the supernatant [illegible] for the amount of agrocin activity. Agrocin activity is expressed [illegible] arbitrary units (A U) which is the [illegible] of the highest dilution [illegible] give a positive zone [illegible] inhibition.

3.4.5 Induction of the bacteriocin

The agrocin was not induced by ultraviolet light or mitomycin C, as indicated in Table 3.4 and 3.5.

3.4.6 Curing of D286 and 396::Tn7 of agrocin production

No D286 or 396::Tn7 colonies were found which had lost the ability to produce agrocin. C58 was not cured of its Ti plasmid; all colonies tested remained sensitive to the agrocin produced by 396::Tn7.

3.4.7 Agarose gel electrophoresis of the plasmids of D286 and D208

The plasmid profiles of *A. tumefaciens* strains D286 and D208 are shown in Figures 3.6a and 3.7a, respectively, and the spectrophotometric scan of the plasmids is shown in Figures 3.6b and 3.7b, respectively. The scan of the plasmids of K84 is shown in Figure 3.8. As can be seen (Figures 3.6a and 3.7a), plasmids of strains D286 and D208 can be extracted using heat treatments of 95°C for up to 6 and 13 mins respectively. Heat treatments for periods longer than this leads to denaturation of the plasmid DNA.

Table 3.4 The effect of mitomycin C on the production of agrocin by D286.[1]

Concentration of mitomycin C (ug/ml)	% survival	Amount of agrocin after time of recovery (arbitrary units)					
		0	24	26	28	30	48 (hrs)
0	100	0	0	0	1	2	4
0.2	23	0	0	0	1	2	8
0.4	4	0	0	0	1	2	4
0.6	0.9	0	0	1	1	2	5
0.8	0.15	0	0	0	0	1	4
1.0	0.07	0	0	0	0	0	3

[1]A log phase culture of D286 was exposed to mitomycin C for 3 hrs. The cells were washed resuspended in fresh LB and allowed to recover for various times, after which the amount of agrocin was determined. D208 was used as the sensitive indicator. The numbers listed are the average results taken from three separate experiments.

Table 3.5 The effect of ultraviolet light on the production of agrocin by D286.[1]

Time of irradiation (sec)	% survival	Amount of agrocin after time of recovery (arbitrary units)					
		0	24	26	28	30	48 (hrs)
0	100	0	0	0	1	2	10
30	58	0	0	0	2	4	10
60	31	0	0	0	1	2	12
90	25	0	0	1	1	1	10
120	12	0	0	1	1	1	10
240	0.02	0	0	2	0	0	8

[1]D286 was irradiated for various times and allowed to recover for various times, after which the amounts of agrocin was determined. The numbers listed are the averages taken from three separate experiments. D208 was used as sensitive indicator strain.

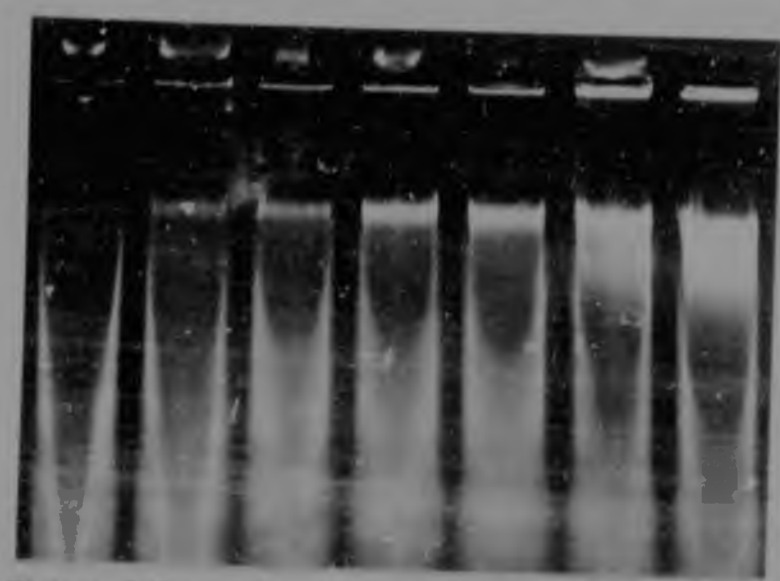
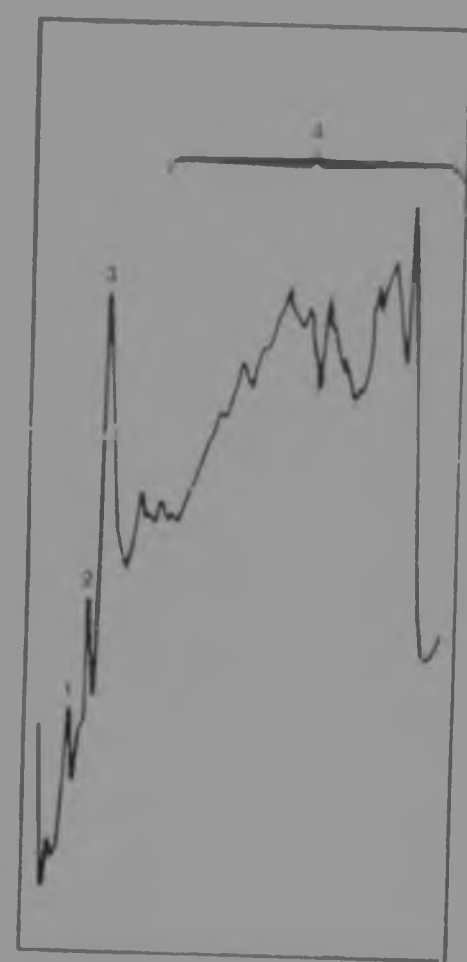


Figure 3.6a. Plasmid profile of D286. Plasmids were extracted at 95°C for various lengths of time



1 Cryptic plasmid
2 deleted Ti or agrocin encoding plasmid
3 chromosomal DNA
4 RNA and degraded DNA

Figure 3.6b. Spectrophotometric scan of the plasmids of D286 extracted at 95°C for 4 min (lane 2).

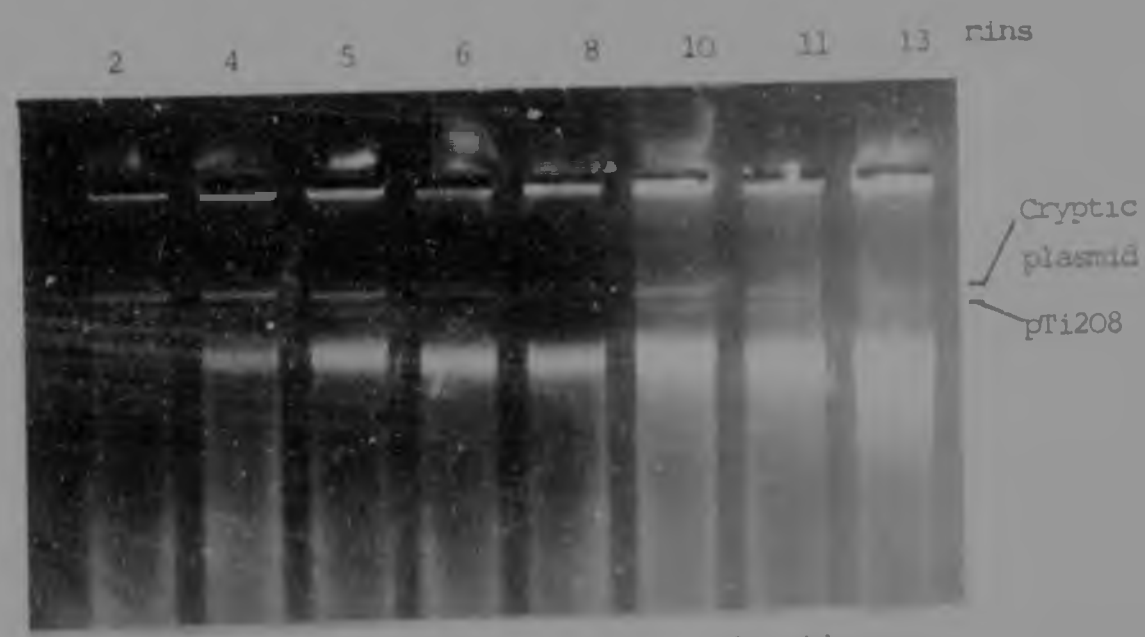


Figure 3.7a. Plasmid profile of the D208. Plasmids were extracted for various lengths of time at 95°C.

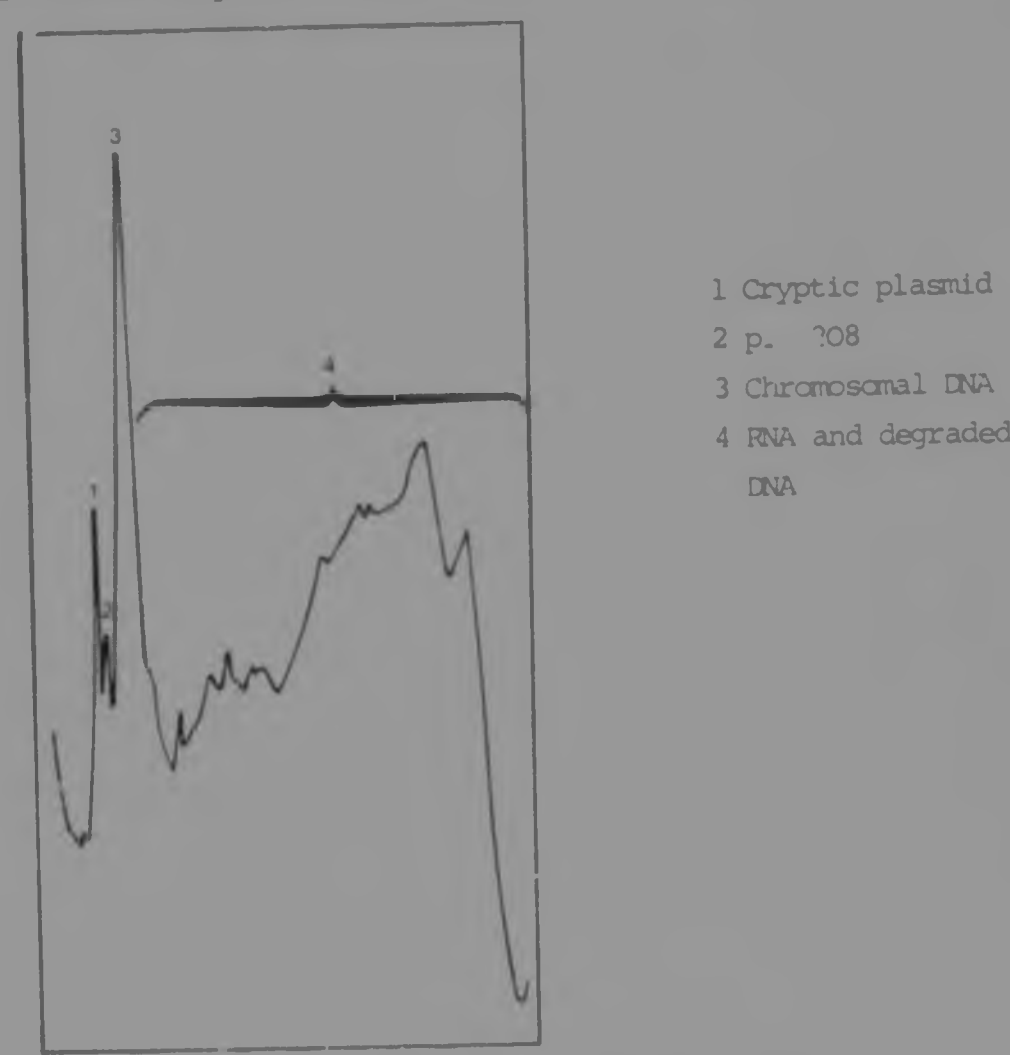


Figure 3.7b. Spectrophotometric scan of the plasmids of D208 extracted at 95°C for 5 min (lane 3)

1 Cryptic plasmid

2 pAt84b

3 pAt84a

Figure 3.8. Spectrophotometric scan of the plasmids of K84 extracted at 95°C for 6 mins.

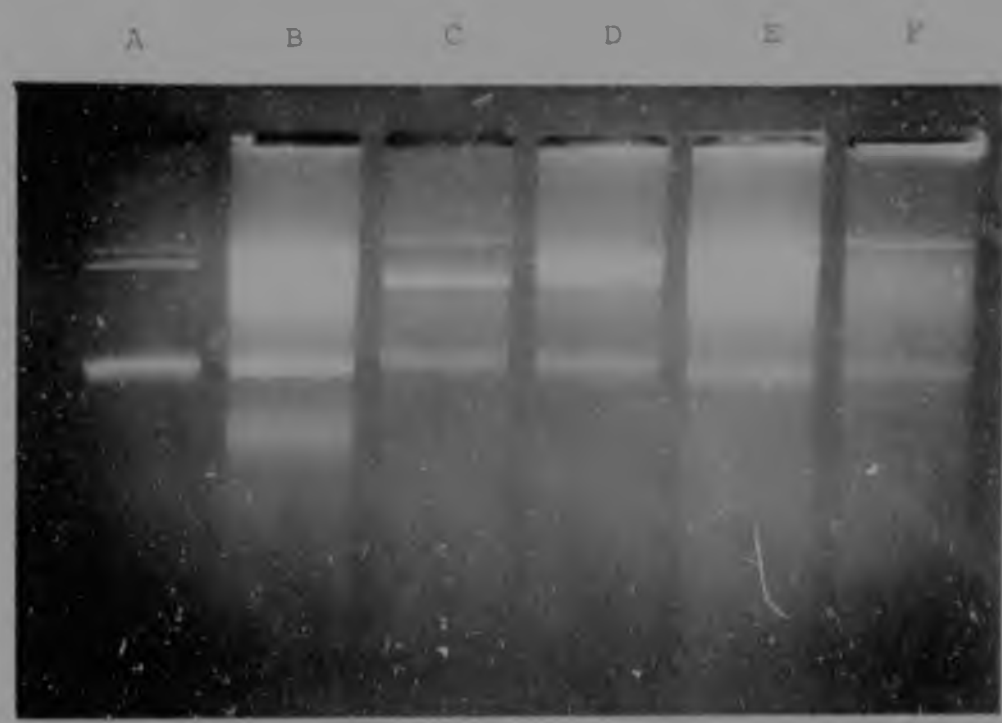


Figure 3.9 Agarose gel electrophoresis of the plasmids of A) K84, B) 396::Tn7, C) D286, D) D208, E) C58, and F) C58Cl. Cleared lysates were subjected to electrophoresis through a 0.7% agarose gel. The gel was strained with ethidium bromide and photographed on a shortwave UV transilluminator.

3.4.8 Effect of the agrocin produced by D286 on *Rhizobium* and *A. rhizogenes* strains

D286 inhibited the growth of *Rhizobium meliloti*, RF14 and a strain from the cowpea miscellany, strain XHH1. The other strains were all resistant to agrocin D286. *A. rhizogenes* strains Tr8.3, Tr1u1 and ATCC11325 were sensitive to agrocin D286. Only strain Tr8.3 was sensitive to agrocin 84 (Table 3.6).

3.4.9 Laboratory tests to determine the effectiveness of agrocin D286 in controlling the formation of crown gall on potato slices

The effect of agrocin D286 on tumor inducing strains is given in Table 3.7. Galls were formed on potato slices inoculated with virulent strains C58, D208 and Ach5. Avirulent strains C58C1 and Ach5C3 did not cause gall formation on potato slices. Neither did the agrocin producing strains D286 and K84. When stationary phase cultures of strain D286 were mixed at different ratios with pathogenic strains harboring nopaline, agropine and octopine plasmids whose growth it had been shown to inhibit *in vitro*, the formation of crown gall tissue on potato slices was inhibited (Table 3.7). Strain K84 inhibited the formation of galls by C58.

Table 3.6 *A. rhizogenes* strains and their sensitivity to agrocins

Strain number	Sensitivity to agrocins from strains		
	D286	396::Tn7	84
15834	R	R	R
Tr1	R	R	R
Tr8.3	S	S	S
Tr101	S	R	R
Tr107	R	R	R
ATCC11325	S	R	R

R, resistant to the agrocin, S, sensitive to the agrocin

Table 3.7 Inhibition of tumor production on potato slices by the use of agrocin producing strains

Strain tested	Cell numbers	Cell numbers of D286	Ratio of tester strain to D286	Tumor formation
C58	2.5×10^9	1.2×10^9	2.1 : 1	-
	5×10^9	1.2×10^9	4.2 : 1	-
	10×10^9	1.2×10^9	8.3 : 1	-
	12×10^9	1.2×10^9	10 : 1	+
D208	1.5×10^9	1.2×10^9	1.25 : 1	-
	3×10^9	1.2×10^9	2.5 : 1	-
	6×10^9	1.2×10^9	5 : 1	-
	9×10^9	1.2×10^9	7.5 : 1	+
Ach5	0.8×10^9	1.2×10^9	1 : 1.5	-
	1.6×10^9	1.2×10^9	1.3 : 1	+
	3.2×10^9	1.2×10^9	2.6 : 1	+
	4.8×10^9	^{9}	4 : 1	+
		Cell numbers of K84	Ratio of tester strain to K84	
C58	6×10^9	5×10^9	1.2 : 1	-
	6×10^9	4×10^9	1.5 : 1	+
	6×10^9	3×10^9	2 : 1	+
C58	5×10^9	Heat killed D286 cells were either added to C58 or D208 cells in equal numbers		+
D208	5×10^9			+

Strains of A. tumefaciens were mixed with D286 cells in different ratios and inoculated on to potato slices. The assay for tumor formation was qualitative. -, no tumor formation on any of the six slices tested; +, tumor formation on all of the six slices tested.

When heat killed strain D286 cells were mixed with equal numbers of C58 and D208 cells, gall formation on potato slices was not inhibited (Table 3.7).

3.5 DISCUSSION

In Luria broth the agrocin of D286 was produced towards the latter part of log phase. There was a 'burst' of agrocin activity in the supernatant at an OD_{540} of 1.36 (32 AU). After this there was a loss of agrocin activity. After 90 hrs incubation there was, however, still a basal level (1 AU) of agrocin present. Thus the agrocin produced by D286 was unstable in the medium in which the producer cells were grown. All the agrocin was located in the extracellular fraction of the culture and was not found intracellularly or bound to the cell.

Since there was a considerable loss of agrocin activity after the 'burst' of production, an attempt was made to identify this agrocin degrading or destabilising substance. It has been suggested (M. van Montagu, personal communication) that the agrocin producing strain may 'degrade' the agrocin as a protective device. Agrocin was incubated at 26°C with the intracellular fraction of washed and unwashed cells (sonicated cells), the cells themselves (washed and unwashed) and with the supernatant of a D286 culture between OD_{540} 0.72 to 1.69. The agrocin was not inactivated by a component of any of these fractions. The reason for the loss of activity in the culture is unknown. However, it

has been suggested(D. R. Woods, personal communication) that a co-metabolite responsible for agrocin inactivation may be produced concomitantly with agrocin production.

A larger zone of inhibition was produced by all three agrocin producing strains D286, K84 and 396::Tn7, against sensitive strains on sodium glutamate plates than on glucose plates. However, this phenomenon cannot be attributed to catabolite repression, since it was not observed in liquid minimal medium. The same levels of agrocin were produced in both sodium glutamate and glucose containing media (32 AU) but agrocin was produced earlier in the growth cycle in sodium glutamate medium (i.e. less cells producing more agrocin in sodium glutamate medium). However, it has been suggested by Moore and Warren(1979) that glucose differentially affects sensitivity of the test strain to agrocin rather than the production of agrocin 84 by strain 84. The same sized zones of inhibition of a particular test strain were produced when glucose at concentrations of 5 to 20 g/l were added to the defined medium. Conversely, these increasing concentrations of glucose caused a progressive decrease in the diameter of inhibition zone of another A. tumefaciens strain around strain 84.

More agrocin was produced at 28°C than 25°C, and more agrocin was produced after 72 hrs incubation compared to 48 hrs incubation. Different agrocin sensitive strains exhibited different degrees of sensitivity. A larger zone of inhibition was constantly observed around D286 when D208 was used as the test strain than when C58 was used as the test strain (Figures 3.3 and 3.4) although both plates were exposed to the same conditions.

Contrary to the findings for other bacteriocins produced by, for example, Serratia, Pseudomonas and Listeria strains(Mayr-Harting et al., 1972), ultraviolet light and mitomycin C did not induce the production of agrocin D286. Curing agents were used in a first step to determine whether D286 was plasmid encoded. The curing agents used were acridine orange, acriflavine, coumermycin, ethidium bromide and mitomycin C. Neither D286 nor 396::Tn7 (which carries the agrocin 84 encoding plasmid, pAt396) were cured of agrocin production. Neither was C58 cured of its Ti plasmid. Lin and Kado(1977) have found that some strains are more easily cured than others and that curing took place progressively within a given cell population. Resistant strains were cured eventually by a greater number of subcultures. With ethidium bromide as the curing agent it was necessary to use very low cell numbers (<100 cells/ ml).

Acridine orange and crystal violet were ineffective in curing strains of their Ti plasmids(Lin and Kado, 1977).

Strain D286 was originally isolated as a pathogenic strain from a crown gall on a eucalyptus tree. However, when it was discovered to produce an agrocin and reinoculated into Datura and tobacco plants (F. van Zyl, personal communication), it was found to have lost its pathogenicity. This could either have been due to the complete loss of an inherently unstable Ti plasmid, or to the deletion of parts of it including the oncogenic functions. As it was of interest to determine which of these had occurred and whether agrocin production might be plasmid determined in strain D286, plasmid screens of D286, 396::Tn7 and K84 were compared. The results are shown in Figures 3.6, and 3.9. Strain D286 can be seen to harbour two plasmids, a cryptic plasmid, which migrated at the same rate as the cryptic plasmid of strain C58C1, C58 and 396::Tn7 (Figure 3.9), and a second plasmid which migrated further than the Ti plasmid of C58 and 396::Tn7, but slower than the agrocin plasmid of 396::Tn7. Hence with this data it is not possible to determine whether this plasmid encodes agrocin production, or whether it is a deleted Ti plasmid. However, if it is an agrocin encoding plasmid, this would explain the

difficulty in curing the plasmid.

Agrobacterium strains were heat treated at 95°C for various lengths of time as it was found that the plasmids from different strains were optimally extracted after different periods of heat treatment. Plasmids could be extracted from strain D286 and D208 after heat treatments of up to 6 and 13 minutes, respectively. Heat treatments for a longer period of time denatured the plasmid DNA.

If strain D286 is to be used in biological control it is essential that it not be active against Rhizobium, the commonly used nitrogen fixing bacterium. D286 agrocin was found to inhibit the growth of only two Rhizobium strains, RF14 and XHH1, neither of which is used commercially in South Africa. However, this sensitivity also indicates the close relationship between Agrobacterium and Rhizobium species. A. rhizogenes strains Tr8.3, Tr 101 and ATCC11325 were sensitive to agrocin D286. This agrocin had a broader spectrum of activity against A. rhizogenes strains than agrocin 84, again indicating its broad host range.

Agrocin D286 had a broad host range in vitro. It was also able to control gall formation (on potato slices) by strains C58, Ach5 and D208, all of which carry a Ti plasmid. The inhibition was effective with an eight fold excess of C58 (Table 3.7) and a five fold

excess of D208. A ratio of 1:1 was, however, required to control gall formation by Ach5. Strain K84 was also found to inhibit gall formation by C58, but only when equal numbers of cells were inoculated. When heat killed D286 cells were mixed with equal numbers of C58 and D208 cells, gall formation on potato slices was not inhibited, indicating that agrocin production by viable cells was necessary for inhibition of gall formation and not merely competition for attachment to plant cells.

It has been suggested that it may be possible to use D286 in combination with strain 84 as a co-inoculum in order to extend the range of biological control. As D286 has been found to grow more rapidly than strain 84 in both Luria broth and minimal medium, competition studies will have to be carried out first with these two strains.

4. PHYSICAL CHARACTERISTICS AND MODE OF ACTION OF THE AGROCIN PRODUCED BY *A. TUMEFACIENS* STRAIN D286.

4.1 SUMMARY

The crude and partially purified agrocin produced by D286 was resistant to protease, DNase I and RNase A. The agrocin was stable over a wide pH range, from 3 to 9 for 22 hrs. Temperatures of 37°C and above inactivated the agrocin. The agrocin produced by *A. tumefaciens* strain D286 inhibited the synthesis of deoxyribonucleic acid, ribonucleic acid and proteins in a sensitive, virulent strain of *A. tumefaciens*, D208. The inhibition of macromolecular syntheses was apparent immediately after the addition of the agrocin.

4.2 INTRODUCTION

It is now generally accepted that the reaction of a bacteriocin with the sensitive cell may be divided into two main phases(Reeves, 1965; Mayr-Harting *et al.*, 1972). The first consists of a rapid, normally irreversible adsorption to specific receptors at the cell surface (adsorption phase). This is followed, some measurable time later, by the appearance of

pathological changes leading eventually to the death of the cell (lethal phase). Whereas the adsorption phase occurs in resting or even dead cells, progression to the lethal phase is energy dependent and its onset may be delayed by use of metabolic inhibitors.

Agrocin 84 produced by A. radiobacter K84 has been shown to inhibit virulent strains of A. tumefaciens harbouring a nopaline Ti plasmid, but not a strain lacking the Ti plasmid(Engler et al., 1975; van Larebeke et al., 1975; Watson et al., 1975). The primary site of action of the agrocin has not yet been determined. Murphy and Roberts(1979) showed that agrocin 84 is actively taken up by a high affinity transport system. At $0^{\circ}C$ no uptake of the agrocin was observed showing that actively metabolising cells are essential for agrocin uptake. Unmetabolised agrocin 84 was transported inside the cell and was associated with the soluble fraction of ruptured bacteria rather than the outer walls or cytoplasmic membrane. It has been proposed that uptake of agrocin is due to the presence of a membrane protein found in the periplasmic space of the sensitive cells(Sonoki and Kado, 1978; Murphy and Roberts, 1979).

It was shown by Das et al.(1978) that agrocin 84

inhibited DNA synthesis without causing DNA degradation. Inhibition occurred after 30 min of treatment of a log phase culture. Protein and RNA syntheses were completely unaffected at low concentrations of agrocin and only partially affected at high concentrations. McCardell and Pootjies(1976), in contrast, demonstrated that all macromolecular syntheses were inhibited with the major effect on DNA synthesis.

The stability of a bacteriocin preparation is of practical importance when choosing methods of physical manipulation. The stability of several bacteriocins has been shown to decrease with increasing purity(Mayr-Harting et al.,1972) and this must be borne in mind when handling these substances. The factors affecting the stability of the bacteriocin are temperature, pH and exposure to various agents such as UV light and chloroform which is used to sterilise the bacteriocin preparations. Most bacteriocins may be lyophilised and recovered without great loss of activity, but exceptions to this generality are known(Mayr-Harting et al., 1972).

Thompson et al.(1977) found that agrocin 84 was resistant to all proteases and peptidases. Some workers have characterised agrocin 84 as acidic, ethanol soluble, heat stable, less than 1000 in

molecular weight, and with reduced biological activity at pH below 4 and above 9. Contrary to this, Kerr found that the biological activity of highly purified agrocin was lost at 21^{0}C to 23^{0}C(see Moore and Warren, 1979).

In light of the above considerations, the characteristics of the crude and partially purified agrocin of D286 were determined. The mode of action of D286 agrocin on the sensitive strain, D208, was also studied.

4.3 MATERIALS AND METHODS

4.3.1 Partial purification of the agrocin produced by strain D286

Agrocin D286 was partially purified by Lizzie Askjaer. Strain D286 was grown in LB at 26^{0}C to OD_{540} 1.36 (as measured on a Spectroplus spectrophotometer) when agrocin activity was maximal (Figure 3.1). The cells were removed from the supernatant by centrifugation at 8500 x g for 10 mins. Charcoal (Riedel-De-Haen Ag Seelze Hannover) at 0.5 g/100 ml was added to the supernatant. The agrocin was absorbed to the charcoal by stirring at 4^{0}C for 24 hrs. The charcoal was removed from the supernatant

by centrifugation at 20000 x g for 15 mins. The agrocin was eluted from the charcoal with two changes of 70% ethanol over 24 hrs at 4°C and concentrated by rotary evaporation at 37°C of the agrocin containing ethanol extract.

The agrocin was separated on a DEAE Sephadex A25 column using a stepwise gradient of 0.05 M to 0.5 M ammonium phosphate pH 6.3. As preliminary tests had indicated that agrocin D286 might be a nucleotide similar to agrocin 84, absorption at 254 nm was monitored and the peaks assayed for agrocin activity. Samples containing partially purified agrocin were concentrated by lyophilisation. Ten millilitres of partially purified agrocin yielded 280 mg (dry weight) after lyophilisation.

4.3.2 Physical characterisation of agrocin D286

4.3.2.1 Temperature stability of the crude and partially purified agrocin

One millilitre samples (4 AU) of the crude D286 agrocin (in LB) as well as the partially purified agrocin (25 mg/ml dry weight in distilled water) were incubated at the following temperatures: 4°C, 25°C, 37°C, 45°C and 60°C. After 0, 3 and 22 hrs incubation at the specified temperature, 200 µl

aliquots were withdrawn from each sample and assayed for the presence or absence of agrocin activity. D208 was used as the agrocin sensitive strain.

4.3.2.2 pH stability of the crude and partially purified agrocin

The stability of the crude agrocin at different pH values was determined by mixing equal volumes of the supernatant of the D286 culture and 50 mM Tris-HCl (with pH values ranging from 2 to 12). The agrocin was incubated at 26°C for 0, 3, 22 and 90 hrs and the pH was checked before assaying. Partially purified agrocin (5 mg/ml; 4 AU) was dissolved in 50 mM Tris-HCl with varying pH values and assayed for agrocin activity.

4.3.2.3 Chemical properties of the crude and partially purified agrocin

DNase I and RNase A (50 µg/ml), and protease (100 µg/ml) were each dissolved separately in 0.02 M Tris pH 8.2. Equal volumes of the crude agrocin and the enzymes were mixed and incubated at 26°C for 0, 3 and 22 hrs before assaying for agrocin activity. Partially purified agrocin (25 mg/ml) was dissolved in the enzyme solutions separately.

The UV absorption spectrum of the partially purified D286 agrocin was compared with that of agrocin 84.

4.3.3 The effect of agrocin 286 on protein and nucleic acid synthesis

The effect of agrocin D286 on nucleic acid and protein synthesis was determined using an adaptation of the method of Friesen(1968). An overnight culture of D208 in LB was diluted 1:10 in LB and grown at 26°C on an orbital shaker until an OD_{540} 0.5, as measured on a Spectroplus spectrophotometer, was reached. The culture was divided in two. Agrocin was added to one of the samples. The cultures were assayed for protein synthesis by the incorporation of ^{14}C-leucine (2 µCi/ml) into trichloroacetic acid (TCA) precipitable material. Samples (0.1 ml) were removed at 30 min intervals, added to 0.1 ml cold 10% TCA (wt/vol)containing 1 mg/ml unlabelled leucine, and kept on ice for 30 min. The samples were filtered on Whatman glass fibre filters (GF/C) and the precipitates were washed twice with 10 mls cold 5% (wt/vol) TCA, and once with 10 ml 1% (vol/vol) acetic acid. The filters were dried, and added to 10 ml scintillation cocktail and counted using a Packard Tricarb scintillation counter model no. 2660.

The effect of agrocin on RNA and DNA synthesis was determined by the incorporation of ^{3}H-adenine (1 µCi/ml) into TCA precipitable material. The procedure used for the determination of total nucleic

acid synthesis is the same as the one used for the determination of protein synthesis except that samples (0.1 ml) were added to 0.1 ml cold 10% TCA (wt/vol) containing 1 mg/ml unlabelled adenine. In order to determine DNA synthesis 0.1 ml 1 M NaOH was added to 0.1 ml samples and incubated overnight at 37°C to hydrolyse RNA and proteins. The samples were then neutralised by the addition of 7.5 µl of 1% (vol/vol) acetic acid. Two hundred microlitres of cold 10% (wt/vol) TCA containing 1 mg/ml unlabelled adenine was added to the samples. The samples were kept on ice for 30 mins and then filtered on to GF/C filters. RNA synthesis was calculated by the difference between total nucleic acid and DNA counts.

In order to confirm that DNA was not hydrolysed upon incubating overnight at 37°C in 1 N NaOH, DNA synthesis was determined by the incorporation of ^{3}H-thymine (1 µCi/ml) into TCA precipitable material. The procedure was the same as that used for the determination of protein synthesis except that 0.1 ml cold 10% TCA containing 1 mg/ml thymine was added to the samples.

The concentration of agrocin used in labelling experiments was 20 mg/ml. This amount of agrocin gave 2 AU of agrocin activity.

4.4. RESULTS

4.4.1 Physical characteristic of agrocin D286

Partially purified and crude agrocin were stable at 4^0C and 26^0C for at least 90 hrs. Both preparations were inactivated after 22 hrs incubation at 37^0C and 45^0C, and after 3 hrs incubation at 60^0C (Table 4.1).

Both crude and partially purified agrocin D286 were stable at pH 3 to 9 after 22 hrs incubation. However, the agrocin was inactivated after 90 hrs at pH 3, 4 and 9 (Table 4.2)

The agrocin preparations were resistant to enzymes DNase I,RNase A and protease (Table 4.3).

The UV absorption spectra of agrocins D286 and 84 are shown in Figure 4.1. Like agrocin 84, agrocin D286 has a maximum absorbance at 264 nm(Roberts *et al*., 1977; Thompson *et al*., 1979).

4.4.2 The mode of action of agrocin D286

Inhibition of DNA, RNA and protein synthesis was detected within 30 mins after the addition of

Table 4.1 The effect of temperature on the activity of agrocin D286 (crude and partially purified).[1]

Temperature °C	Activity after incubation at the specified temperature for		
	0 hrs	3 hrs	22 hrs
4	+	+	+
26	+	+	+
37	+	+	-
45	+	+	-
60	+	-	-

[1]The agrocin was incubated at the specified temperature for various lengths of time before being assayed for agrocin activity.
+: agrocin is active
-: agrocin is inactive

Table 4.2 The effect of pH on the activity of agrocin D286 (crude and partially purified)[1]

pH	Time of incubation at 26°C			
	0 hrs	3 hrs	22 hrs	90 hrs
2	+	-	-	-
3	+	+	+	-
4	+	+	+	-
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	-
10	+	-	-	-
11	+	-	-	-
12	+	-	-	-

[1]The crude agrocin (in LB) was diluted 1:1 with the different pH buffers to yield the final pH listed. Partially purified agrocin (25 mg/ml) was dissolved in the pH buffers. The agrocin was incubated for various lengths of time before being assayed for agrocin activity. +: agrocin is active, -: agrocin is inactive

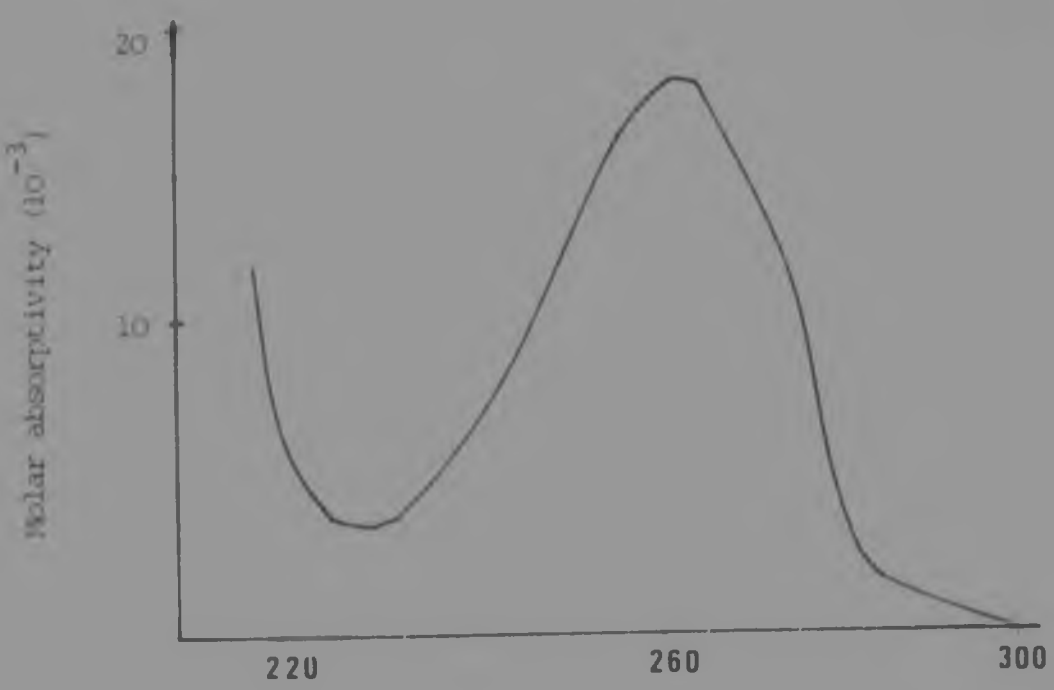
Table 4.3 The effect of DNase I, RNase A and protease on the activity of agrocin D286 (crude and partially purified)[1].

Enzyme	Time of incubation at 26°C		
	0 hrs	3 hrs	22 hrs
DNase I (50ug/ml)	+	+	+
(100ug/ml)	+	+	+
RNase A (50ug/ml)	+	+	+
(100ug/ml)	+	+	+
Protease (100ug/ml)	+	+	+
(200ug/ml)	+	+	+

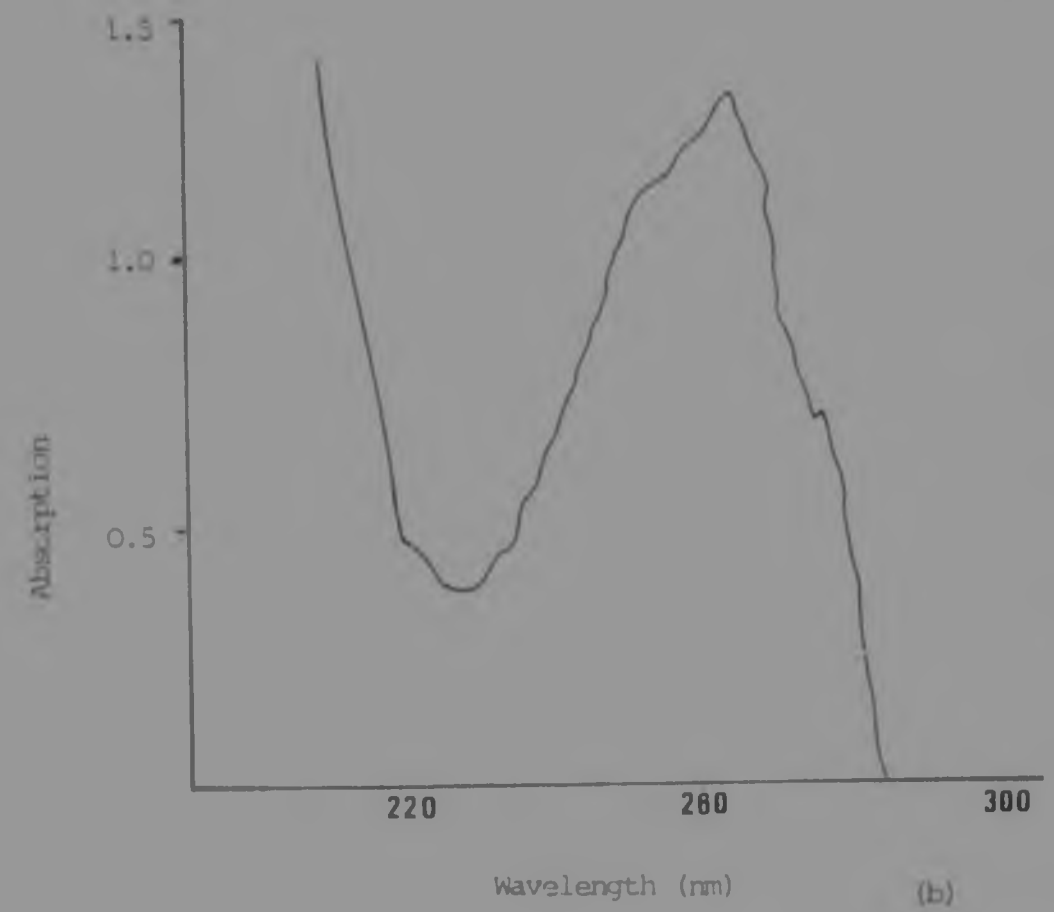
[1]The agrocin was exposed to the enzymes for various lengths of time before being assayed for agrocin activity.

+: agrocin is active

-: agrocin is inactive



(a)



(b)

Figure 4.1(a) Spectrophotometric absorption of agrocin 84 (from Tate et al., 1979)

(b) Spectrophotometric absorption of agrocin D286

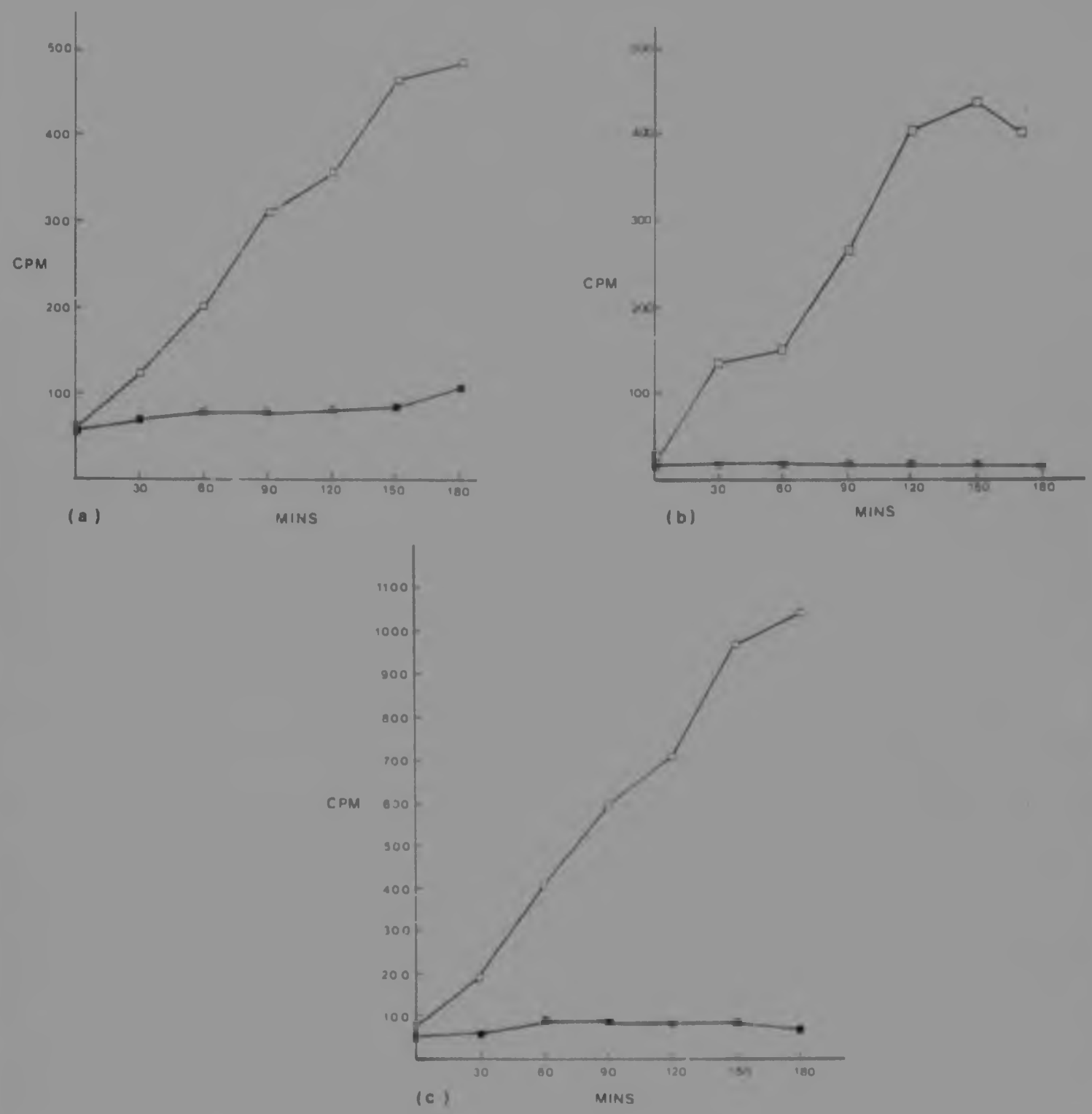


Figure 4.2(a). The effect of D286 agrocin on DNA synthesis of sensitive D208 cells. Incoporation of [^{14}C]-[illegible] into [illegible] cells [illegible] and [illegible] cells to which agrocin had been added (■-■) was determined.

(b). The effect of D286 agrocin on RNA synthesis of sensitive D208 cells. Incorporation of [^{14}C]-adenine [illegible] into [illegible] cells [illegible] and [illegible] cells to which agrocin had been added (■-■) was determined.

(c). The effect of D286 agrocin on protein synthesis of sensitive D208 cells. Incorporation of [^{14}C]-leucine [illegible] into [illegible] cells [illegible] and [illegible] cells to which agrocin had been added (■-■) was determined.

Samples were taken at intervals, added to $10^{[illegible]}$ TCA containing 1mg/ml thymine, adenine or leucine, respectively, filtered on to glass fibre filters, washed, and counted.

partially purified D286 agrocin. This was measured by the incorporation of ^{3}H-thymine, ^{3}H-adenine and ^{14}C-leucine, respectively, in sensitive D208 cells (Figure 4.2).

4.5 DISCUSSION

The physical characteristics of agrocin D286 were studied as these will be useful in determining the physical constraints to which the agrocin may be exposed.

Agrocin D286 was stable at 26^{0}C but unstable at 37^{0}C and temperatures above this. It may be possible that agrocin is produced by D286 at temperatures above 26^{0}C but is not detected due to inactivation of the agrocin.

Agrocin D286 exhibited considerable pH stability being stable over a pH range of 3 to 9 after 22 hrs. After 90 hrs the agrocin was inactivated at pH 3, 4 and 9.

The exact chemical nature of D286 agrocin is unknown. However, due to its UV-spectrum (Figure 4.1) and since it is not inactivated by DNase I, RNase A and protease (Table 4.3), it is possible that it is a

partially purified D286 agrocin. This was measured by the incorporation of ^{3}H-thymine, ^{3}H-adenine and ^{14}C-leucine, respectively, in sensitive D208 cells (Figure 4.2).

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The exact chemical nature of D286 agrocin is unknown. However, due to its UV-spectrum (Figure 4.1) and since it is not inactivated by DNase I, RNase A and protease (Table 4.3), it is possible that it is a

nucleotide analogue similar to agrocin 84. The agrocin of D286 was partially purified by Lizzie Askjaer and its UV-absorption spectrum is consistent with its being a nucleotide.

D286 agrocin was shown to inhibit DNA, RNA and protein synthesis of sensitive D208 cells. The effect of the agrocin on macromolecular synthesis was observed immediately after the addition of the agrocin. The agrocin may inhibit DNA synthesis first, and then only secondarily affect RNA and protein synthesis. It has been suggested that the toxic action of agrocin 84 may be similar to the blockage of pyrimidine and purine synthesis by adenosine and its analogues(Moore and Warren, 1979). It has been suggested(Murphy, Tate and Kerr, 1981) that the fraudulent nucleotide core of agrocin 84 is incorporated into the DNA of sensitive bacteria. This nucleotide core lacks a 3'-OH group, and could act as a chain terminator and thereby inhibit DNA replication. Our results differ from those of McCardell and Pootjies(1976) who showed that RNA and protein synthesis of strain H38-9 were drastically inhibited by agrocin 84, and DNA synthesis inhibited to a lesser extent. Results also differ from those of Das _et al_.(1979) who showed that DNA synthesis was primarily affected by agrocin 84, and that protein and RNA synthesis was only affected when a high

concentration of agrocin was added to the cell culture. The exact mechanism of inhibition of growth of sensitive cells by agrocin is not proven even for agrocin 84. However, the overall similarities observed between agrocins 84 and D286 lead to the conclusion that they may well inhibit the growth of sensitive cells in similar manners.

5. LOCALISATION OF THE AGROCIN SENSITIVITY REGION ON THE PHYSICAL MAP OF THE NOPALINE TI PLASMID

5.1 SUMMARY

A series of mutants at various sites on pTiC58, a nopaline Ti plasmid, were tested in order to localize the region on pTiC58 encoding sensitivity to the agrocin produced by strain D286. These mutants were deletion mutants and were derived by insertion of Tn1, Tn7 or RP4(Holsters *et al.*, 1980). Sensitivity to agrocin D286 was localised to 11 - 18 Md on pTiC58. Mutants abolishing agrocin D286 sensitivity mapped in a region which was found to coincide with the region abolishing exclusion of bacteriophag AP1(Holsters *et al.*, 1980).

The agropine Ti plasmid of strain D208 was found to be responsible for agrocin D286 sensitivity.

5.2 INTRODUCTION

In order to study the functional organisation of the nopaline plasmid, pTiC58, and the octopine plasmid, pTiB6S3, mutants of the plasmids were isolated and characterised(Holsters *et al.*, 1980; De Greve *et al.*, 1981). The transposons Tn1(Hedges and Jacob,

1974) and Tn7(Barth *et al.*, 1976), and the P type plasmid RP4(Datta *et al.*, 1971) which can insert into the Ti plasmid(Holsters *et al.*, 1979b and 1980) were used to derive deletion and insertion mutations in pTiC58. Tn7 was used to derive insertion and deletion mutations in pTiB6S3(De Greve *et al.*, 1981).

Transposons are important tools in plasmid genetics. They mutate by direct insertion and can exert a polar effect on distal genes. They can also promote deletions(Kleckner, 1977). The presence of the transposon in the bacterium can be detected since transposons confer antibiotic resistance on the host bacterium. For example, Tn7 encodes resistance to the antibiotics streptomycin, spectinomycin and trimethoprim. Large plasmids, such as the Ti plasmid of *A. tumefaciens* presumably contain many different sites into which transposons can insert, thereby giving rise to alterations of a known plasmid phenotype(Hernalsteens *et al.*, 1978; Dhaese *et al.*, 1979).

The mutant plasmids were assayed for Ti plasmid determined properties. Mutants were isolated which had lost oncogenic properties (*Onc*$^-$), phage AP1 exclusion (*Ape*$^-$), the ability to synthesize octopine and nopaline (*Ocs*$^-$ and *Nos*$^-$), the ability to catabolize octopine and nopaline (*Occ*$^-$ and *Noc*$^-$),

the ability to catabolize agropine (Agc$^-$), sensitivity to agrocin 84 (Ags^R) and the conjugative property (Tra$^-$). The actual mapping of the mutations required a physical map of the Ti plasmids. Physical maps have been determined for the nopaline plasmid, pTiC58 (Depicker et al., 1977 and 1980b; Holsters et al., 1980) and for the octopine plasmid pTiAch5 (De Vos et al., 1981) using restriction enzymes. It has been shown by restriction digest patterns and electron microscopy that the octopine Ti plasmids pTiAch5 and pTiB6S3 are nearly identical (Engler et al., 1977; Genetello et al., 1977). Mutants that inactivated most of the known Ti plasmid phenotypes were then located on the physical map of the plasmids. The location of the transposon insertion sites on a plasmid map was actually achieved by isolating plasmid DNA from the mutant strain, followed either by analysis of its restriction endonuclease digest pattern or electron microscope studies of heteroduplex molecules. Restriction fragments were separated on an agarose gel, denatured and transferred to a nitrocellulose filter. These were hybridised against the appropriate purified wild type plasmid, either pTiC58 or pTiB6S3 or against cloned segments of the plasmids (Holsters et al., 1980; De Greve et al., 1981).

DNA sequences homologous to the probe were detected

by autoradiography, thus generating a restriction endonuclease pattern of the plasmid from a digest of total bacterial DNA. Mutant fragments were readily identified by their different positions compared to a wild type reference(Dhaese et al., 1979; Holsters et al., 1980; De Greve et al., 1981).

In this way it was possible to map the position of sensitivity to agrocin 84 on the nopaline plasmid pTiC58. Two independently isolated Tn7 insertions in pTiC58 at position 87 Md were found to inactivate the agrocin sensitivity phenotype. Agrocin sensitivity has therefore been allocated to position 87 Md on the pTiC58 map(Holsters et al., 1980)(see Figure 1.1).

It is surprising that through evolution genes still exist which code for the uptake of toxic substances like the agrocins. Agrocinopines are phosphorylated sugars which share several biological properties with the opines(Ellis and Murphy, 1981). It has been found that the agrocinopines are the "legitimate" substrates for the permease which also allows uptake of agrocin 84. Agrocin 84 permease is normally partially expressed and is fully induced by the agrocinopines. Agrocin 84 has therefore "pirated" the system of uptake of agrocinopines. Consequently, there was competitive uptake of agrocin and the agrocinopines by specific Ti plasmid containing

bacteria(Ellis and Murphy, 1981).

The agrocin produced by strain D286 has been found to inhibit the growth of Agrobacterium strains harboring the nopaline, octopine and agropine type Ti plasmids. In an attempt to localize the region encoding agrocin sensitivity on pTiC58, mutants of pTiC58 with deletions in various regions were assayed for sensitivity or resistance to the agrocin produced by strain D286.

5.3 MATERIAL AND METHODS

5.3.1 Bacterial strains

Bacterial strains used, as listed in Table 5.1 were kindly supplied by M. van Montagu, Ghent.

5.3.2 Assay for bacteriocin sensitivity or resistance

Strains carrying the mutant plasmids were screened for resistance or sensitivity to the agrocins produced by strains D286, 396::Tn7 and K84 using the method of Kerr and Htay(1974)(see section 2.3.2).

Table 5.1 Characteristics of deletion mutants in the nopaline plasmid pTiC58 (from Holsters *et al.*, 1980)

Plasmid number	Deletion in pTiC58 generated by	Phenotype of nopaline plasmid
4005	RP4	$Agr^{R}Ape^{-}$
4001	RP4	$Agr^{R}Ape^{-}$
3196	Tn7	$Agr^{R}Tra^{-}$
3178	Tn1	None
3170	Tn1	None
3101	Tn7	$Noc^{-}Arc^{-}Orc^{-}Nos^{-}$
3190	Tn7	$Noc^{-}Arc^{-}Orc^{-}Tra^{-}$
3116	Tn7	$Noc^{-}Arc^{-}Orc^{-}$
3819	Unknown	$Noc^{-}Arc^{-}Orc^{-}Tra^{-}$

bacteria(Ellis and Murphy, 1981).

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3196	Tn7	$Agr^{R}Tra^{-}$
3178	Tn1	None
3170	Tn1	None
3101	Tn7	$Noc^{-}Arc^{-}Orc^{-}Nos^{-}$
3190	Tn7	$Noc^{-}Arc^{-}Orc^{-}Tra^{-}$
3116	Tn7	$Noc^{-}Arc^{-}Orc^{-}$
3819	Unknown	$Noc^{-}Arc^{-}Orc^{-}Tra^{-}$

5.3.3 Curing of the agropine Ti plasmid of D208

Strain D208 was passaged at 37°C on YMA in order to cure it of its Ti plasmid. After eight passages, the resulting isolate was assayed for agrocin D286 sensitivity. The strain was screened for the presence of plasmids as described in section 3.3.8. The strain was tested for virulence on potato slices as described in section 3.3.10.

5.4 RESULTS

A summary of the effect of the agrocin produced by D286, 396::Tn7 and K84 on the deletion mutants of pTiC58 is given in Table 5.2.

Sensitivity to agrocin D286 is encoded by the Ti plasmid in strain D208. Subculturing this strain at 37°C resulted in curing of the Ti plasmid as shown by plasmid profiles (Figure 5.1), loss of sensitivity to agrocin D286 and the loss of virulence as determined by tests on potato slices.

Table 5.2 Nopaline Ti plasmid mutants and their resistance or sensitivity to the agrocins produced by D286, 396::Tn7 and K84[1]

Plasmid number	Approximate map position of deletion (Md)	Effect of agrocin produced by		
		D286	396::Tn7	K84
4005	69 - 130	S	R	R
4001	64 - 130	S	R	R
3196	82 - 115	S	R	R
3178	98.2 - 102.4	S	S	S
3170	125 - 130	S	S	S
3101	131.2 - 11	S	S	S
3190	99 - 13.2	R	S	S
3116	6 - 17.4	R	S	S
3819	5 - 18	R	S	S
Control strains				
C58		S	S	S
C58C1		R*	R	R
Ach5		S	R	R
Ach5C3		R*	R	R
C58C1pTiAch5		R	R	R
B6S3		R	R	R
C58C1pTiB6S3		R	R	R
D208		S	R	R
D208C1		R	R	R

[1]Agrocin producing strains were patched on to MM + sodium glutamate plates. After growth, individual mutants were seeded on to the plates. R, resistance to the agrocin, S, sensitivity to the agrocin; R*, faint zone of inhibition.

Table 5.2 Nopaline Ti plasmid mutants and their resistance or sensitivity to the agrocins produced by D286, 396::Tn7 and K84[1]

Plasmid number	Approximate map position of deletion (Md)	Effect of agrocin produced by		
		D286	396::Tn7	K84
4005	69 - 130	S	R	R
4001	64 - 130	S	R	R
3196	82 - 115	S	R	R
3178	98.2 - 102.4	S	S	S
3170	125 - 130	S	S	S
3101	131.2 - 11	S	S	S
3190	99 - 13.2	R	S	S
3116	6 - 17.4	R	S	S
3819	5 - 18	R	S	S
Control strains				
C58		S	S	S
C58C1		R*	R	R
Ach5		S	R	R
Ach5C3		R*	R	R
C58C1pTiAch5		R	R	R
B6S3		R	R	R
C58C1pTiB6S3		R	R	R
D208		S	R	R
D208C1		R	R	R

[1]Agrocin producing strains were patched on to MM + sodium glutamate plates. After growth, individual mutants were seeded on to the plates. R, resistance to the agrocin, S, sensitivity to the agrocin; R*, faint zone of inhibition.

Figure 5.1 Plasmid profiles of D208 (lanes 1 - 6), and D208C1 (lanes 7 - 10). Plasmids were extracted at 95°C for various lengths of time, lanes 1 and 7, 2 min; lanes 2 and 8, 4 min; lanes 3 and 9, 5 min; lanes 4 and 10, 6 min; lane 5, 8 min; lane 6, 10 min.

5.5 DISCUSSION

Sensitivity to the agrocin produced by D286 is plasmid encoded in strain C58 since it has been found that strains cured of the nopaline Ti plasmid pTiC58 are resistant to agrocin D286. A series of mutants at various sites on pTiC58 were tested in order to localize the region encoding sensitivity to agrocin D286.

Strains harbouring the mutant plasmids 4005, 4001, 3196, 3178, 3170 and 3101 which have deletions within the region 64 to 11 Md were all sensitive to agrocin D286. However, the strain harbouring plasmid 3190 which is deleted in the region from 99 to 13.2, was resistant to agrocin D286. Deletion of the 13 to 18 Md region also resulted in resistance to agrocin D286. Therefore the left hand end of the region encoding agrocin D286 sensitivity must lie between 11 and 13 Md. The region up to 18 Md encode agrocin sensitivity. Since plasmids carrying deletions beyond 18 Md were not tested, the right hand border of the gene(s) encoding agrocin D286 sensitivity is not defined. Strains harbouring plasmids 4005, 4001 and 3196 which have been deleted in the regions 69-130, 64-130 and 82-115 Md, respectively, were resistant to agrocin 84 produced by strains 396::Tn7 and K84. These results

are consistent with those of Holters *et al.* (1980) who allocated agrocin sensitivity on pTiC58 at 87 Md.

The regions of the Ti plasmid, pTiC58 where sensitivity to D286 agrocin mapped also encode the Ti plasmid determined exclusion of phage AP1. Whether these functions are associated has not been determined.

It is of interest to note that the region of pTiC58 where agrocin D286 sensitivity maps falls within one of the homologous regions (B region, Figure 1.3) between pTiC58 and pTiB6S3.

The above results (Table 5.2) show that sensitivity to agrocin D286 is encoded by the nopaline Ti plasmid, pTiC58, the octopine plasmid, pTiAch5, and the agropine plasmid, pTiD208. However, strains cured of the former two plasmids, show faint zones of inhibition.

Agrocin 84 may be broken down into an N^{6} and a 5' phosphoramidate substituent (Murphy *et al.*, 1981). It has been found that the N^{6} phosphoramidate substituent of agrocin 84 is responsible for the uptake of agrocin 84. This nucleotide fragment is a competitive inhibitor for the uptake of agrocin 84 *in vivo* and is selectively transported into a sensitive

cell at a rate comparable with agrocin 84, but unlike agrocin 84, it is non-toxic. The 5' phosphoramidate substituent is taken up by both sensitive and insensitive strains and is toxic to both strains. Tate *et al*.(1979) have described a biologically active degradation product of agrocin 84 lacking the N^6 phosphoramidate constituent. This breakdown product had a broad antibiotic spectrum against many strains of *A. tumefaciens* and *A. radiobacter*, and unlike agrocin 84, sensitivity to this product was not determined by the nopaline Ti plasmid. The product inhibited the growth of strain C58C1 and not C58C1(pAt84a)(Ellis *et al*., 1982b). These facts may be relevant to the slight sensitivity to agrocin D286 shown by some non-pathogenic strains i.e. a breakdown product of agrocin D286 may be taken up by C58C1 and Ach5C3 and inhibit the growth of both strains. However the faint zone of inhibition may also be attributed to a non-specific uptake system which is not Ti plasmid encoded.

The possibility of chromosomal involvement in sensitivity to agrocin D286 in some strains is perhaps supported by the unexpected finding that the octopine plasmids pTiAch5 and pTiB6S3 when introduced to C58C1 are unable to render the cells sensitive to agrocin D286.

It has been shown that agrocin 84 has "pirated" an uptake system for agrocinopines in nopaline producing tumors. Similarly, there may well be in tumors produced by octopine and agropine Ti plasmids another type of opine whose uptake system has been pirated by agrocin D286.

6. MOBILISATION OF THE AGROCIN ENCODING PLASMID, pAt396, USING THE CONJUGATIVE PLASMID RP4

6.1 ABSTRACT

The Inc-P1 type plasmid, RP4, was used to mobilise the agrocin encoding plasmid pAt396 from *Agrobacterium* to *Rhizobium* strains, the latter being benign soil organisms. A number of steps were carried out in order to determine whether pAt396 could be mobilised either as a cointegrate RP4::pAt396 plasmid, or as a separate entity from RP4.

RP4 was transferred from *Escherichia coli* J53(RP4) to *A. tumefaciens* 396::Tn7(pAt396). Five 396::Tn7(RP4)(pAt396) transconjugants were used as donors in a cross with C58rif^R and C58Clrif^R to determine whether RP4 could mobilise pAt396. C58rif^R and C58Clrif^R recipients were used to determine whether the Ti plasmid affected the stability and expression of pAt396. The frequency of transfer per donor and recipient of RP4 was 10^{-} and of Tn7 was 10^{-4}. The transfer of pAt396 occurred in 1.6 to 2.6% of transconjugants regardless of whether the Ti plasmid was present or absent in the recipient

strain. Five of the resultant transconjugants, three C58rif^R::Tn7(RP4)(pAt396) and two C53Clrif^R ::Tn7(RP4)(pAt396) strains, were crossed with C58nal^R. This was to determine whether any contained an RP4::pAt396 cointegrate. RP4 and Tn7 were transferred at frequencies of 10^{-1} per donor and recipient. As all transconjugants carried both RP4 and Tn7, it is likely that at this stage Tn7 became transposed on to RP4. In four of the crosses coinheritance of RP4 and pAt396 varied from 2.2 to 11%. In one of the crosses 100% coinheritance of pAt396 and RP4 was achieved. This strain which potentially carried a cointegrate plasmid was used as a donor in a conjugation with C58ery^R. Four isolated colonies of C58nal^R::Tn7(RP4)(pAt396) were used in parallel experiments. RP4 and Tn7 were co-transferred at a rate of 10^{-4} per donor and recipient. However, only 0.5 to 11% of the transconjugants coinherited pAt396, which indicated that a cointegrate RP4::pAt396 plasmid had not been formed. However, RP4 was still capable of mobilising pAt396 at a significant frequency. All these crosses were later confirmed by performing plasmid screens on the donors, recipients and transconjugants.

The C58rif^R::Tn7(RP4)(pAt396) strain (number 3, Table 6.2) which mobilised pAt396 to C58nal^R with 100% efficiency was crossed with ten different recipients

belonging to the Rhizobium genus. RP4 was transferred at different rates in each cross. Out of 5000 transconjugants tested, only five produced agrocin. All of these were Rhizobium meliloti strains. These results indicate that RP4 can mobilise pAt396 into Agrobacterium and into Rhizobium, although in the latter case at extremely low frequencies which may be due to instability of pAt396 in Rhizobium.

Tests to determine whether the Rhizobium transconjugants were effective in inhibiting gall formation by C58 on potato slices were carried out. The Rhizobium transconjugants did not prevent gall formation and therefore appear to be unlikely candidates for the prevention of crown gall disease in plants.

6.2 Introduction

A. radiobacter strain 84 has been used successfully in the biological control of crown gall in many countries. The mechanism of control is by the production of an agrocin which can seletively inhibit many strains of pathogenic Agrobacteria(Kerr 1980). The production of agrocin 84 is encoded by a plasmid,

pAt84a (30Md). Biological control of crown gall is limited to pathogenic strains of A. tumefaciens carrying a nopaline Ti plasmid.

With any entirely new method of disease control, it is always possible that something will go wrong. For example, the pathogenic bacteria might mutate and no longer be subject to control. It is possible that such resistant strains could become more common when inoculation with strain 84 is widely practised(Kerr, 1980).

An even more dangerous situation has been described by Panagopoulos et al. (1979). In field experiments in Greece in which peach seedlings were inoculated with a 1:1 mixture of strain 84 and a pathogenic strain, many galls developed. Pathogenic, agrocin 84 producing strains were isolated from these galls. All agrocin producing strains examined were found to be resistant to agrocin 84 and therefore could not be controlled by strain 84. These characteristics were found in 16.5% of the isolates. It was concluded that the genes controlling agrocin 84 production and resistance could be transferred from strain 84 to a pathogenic recipient. This was confirmed by carrying out crosses between strain 84 and pathogenic Agrobacteria under controlled laboratory conditions(Ellis and Kerr, 1979). Conjugation could

only occur in the presence of nopaline, as nopaline derepressed the genes controlling conjugation and plasmid transfer. During conjugation the agrocin encoding plasmid, pAt84a could be co-transferred with the conjugative plasmid encoding nopaline catabolism, pAt84b. Such a cross is represented diagramatically in Figure 6.1.

Three plasmids are involved: two in strain 84 and one in the pathogen. Strains that acquire a plasmid after conjugation are called plasmid transconjugants. The six possible types of transconjugants are represented in Figure 6.1, and all were isolated. Transconjugant B and C represent potential hazards; they are pathogenic, produce agrocin 84 and are resistant to agrocin 84. They appear to be of the type isolated by Panagopoulos *et al*.(1979).

Despite these problems, biological control in Australia has been nearly 100% effective(Kerr, 1980). There was little or no gall tissue present and no indication of plasmid transfer. It is possible that in Greece, artificial inoculation with pathogens might have resulted in excessively high numbers of virulent bacteria on and around the roots and strain 84 might not be effective under these conditions. Hence galls could develop, and the nopaline produced could facilitate plasmid transfer.

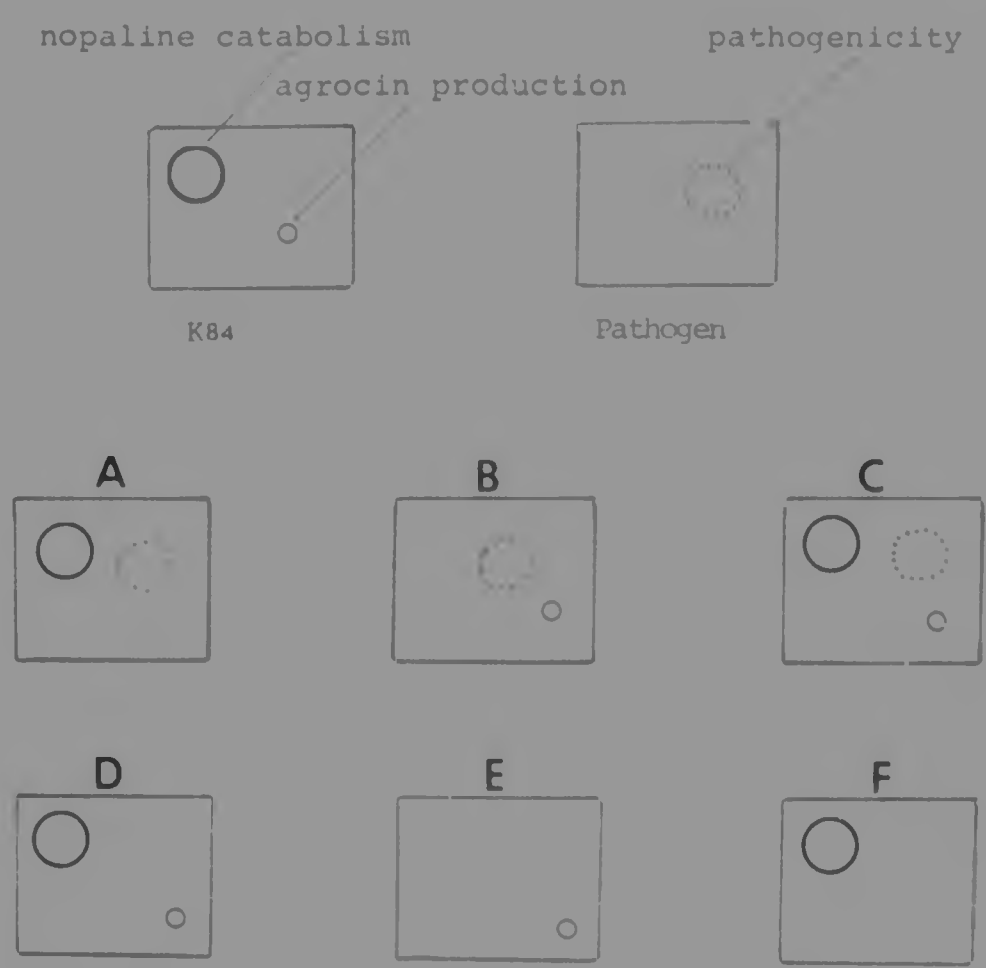


Figure 6.1 Diagrammatic representation of a cross between strain K84 and a pathogenic recipient of Agrobacterium tumefaciens. Chromosomes are not shown. Strain 84 contains two plasmids, one (small circle) coding for agrocin 84 production and resistance to agrocin and the other (large circle) coding for nopaline catabolism and conjugation. The pathogen has one plasmid (dotted line) that codes for pathogenicity and agrocin 84 sensitivity. Six different types of transconjugants can be derived from this cross (from Kerr 1980).

Incomplete biological control has also been attributed to a number of other causes(Panagopoulos *et al*., 1979) such as the level of soil infection, the ratio.of pathogen to non-pathogen, the uneven distribution of the pathogen and strain 84 in the vicinity of the developing seedling and the presence of bacteriocin resistant pathogens(Kerr, 1971; New and Kerr, 1972; Moore, 1977).

Can anything be done to minimize the risk of a breakdown in the effectiveness or biological control of crown gall? One possibility is the isolation of a variant of strain 84 with a defective plasmid transfer or mobilisation system(Kerr, 1980). However, another approach to increasing the chances of successful biological control is the transfer of the ability to produce the effective agrocin to bacteria well adapted for growth at the root surface of the host plant. Such an approach could also prevent the formation of hazardous Agrobacteria such as those represented in B and C of Figure 6.1. Various conjugative plasmids with wide host range could be used to mobilize agrocin production. For example, R factors such as R68.45 with its high potential for mobilization of chromosomal genes(Haas and Holloway, 1978) and RP4(Chilton *et*. *al*. 1976; van Larebeke *et*. *al*. 1977) which can mobilize other

plasmids offer the possibility of gene transfer between different genera of Gram negative bacteria. Such studies should not only help to extend the biological control of crown gall but also to increase the prospects of controlling other soil borne diseases by this method(Ellis and Kerr, 1979).

RP4 is a high molecular weight(3.6 x 10^{6} D) plasmid of the P incompatibility group. It specifies resistance to kanamycin, ampicillin, carbenicillin and tetracycline(Datta *et al*., 1971). The Inc P-1 plasmids, which also include R68 and R68.45 are particularly useful since they possess the ability to establish themselves in a wide range of Gram negative bacteria, as well as having chromosomal mobilising ability (Cma)(Holloway, 1979). However, mobilisation by RP4 is not always efficient. The mobilising ability of RP4 appears to be strain (or species) specific(Beringer and Hopwood, 1976). Three types of mobilisation by plasmids can be distinguished. The first includes those situations in which transfer of the chromosome is not necessarily accompanied by inheritance of the whole plasmid. For example, it has been suggested by Leemans *et al*.(1981) that the non-conjugative plasmid may contain an origin of transfer (*ori T*) and an activation gene (*bom*) but lack trans-acting functions necessary to activate these genes. These functions can be provided by an

appropriate conjugative plasmid. The second type encompasses the F-prime (F') and R-prime (R') examples, and involves the formation of a hybrid plasmid comprising both conjugative plasmid and chromosomal DNA (or non-conjugative plasmid DNA) in which all or most of the plasmid functions have been maintained. Two alternatives occur in this case: (a) both the conjugative and the non-conjugative plasmid may contain an homologous region, which may allow the two plasmids to fuse transiently. Both plasmids are transferred as a cointegrate, (b) if one of the plasmids contains a transposable element, a rec$^+$ independent plasmid fusion can occur and the plasmids are transferred as a cointegrate. Upon resolution of the cointegrate, a copy of the transposable element remains in both plasmids.

RP4 has been shown to mobilize Ti plasmids independently of the pTi-specific conjugation system (often as a pTi::RP4 cointegrate) and RP4 has been shown to be stably maintained in *Agrobacterium* (Chilton *et al.*, 1976; Hooykaas *et al.*, 1977; van Larebeke *et al.*, 1977). Several deletion derivatives and dissociation products of Ti plasmid::RP4 cointegrates have been analysed. Restriction endonuclease analysis of the cointegrates indicated that a specific 1.2 Md segment of RP4 is duplicated and present at both junctions between RP4

and pTi DNA. The genetic properties of the duplicated sequence (IS8) on RP4 suggest that it is an insertion sequence(Depicker *et al.*, 1980a). It has been postulated that insertion of RP4 proceeds by means of transposition of the IS8 sequence to the Ti plasmid. The integration was found to be non-specific with respect to the host target sequences(Depicker *et al.*, 1980a).

It has been shown that members of the genus *Agrobacterium* are able to conjugate with those of the genus *Rhizobium*. The Ti plasmid of *A. tumefaciens* was transferred to different *Rhizobium* species(Hooykaas et al., 1977 and 1981). However the ability to be stably maintained and expressed in *Rhizobium* appeared to be species specific. The Ti plasmid was expressed in *R. trifolii* and *R. leguminosarum*, but not in *R. meliloti*. The Sym plasmid of *R. trifolii* which carries genes determining host range of root nodulation, the infection process, and nitrogen fixation was transferred to *A. tumefaciens*. *A. tumefaciens* transconjugants were able to induce nitrogen fixing root nodules, indicating that the *R. trifolii* Sym plasmid can be expressed in this more distant host.

With the aim of constructing a benign soil bacterium capable of producing agrocin 84, an attempt was made

to transfer the agrocin plasmid of strain 396::Tn7 using the conjugative plasmid RP4 to *Rhizobium* species. We chose members of the *Rhizobium* genus as recipients because of their close relationship to *A. tumefaciens* and because they are rhizosphere bacteria capable of colonizing plants sensitive to *A. tumefaciens* (Vincent *et al.*, 1974; Parker *et al.*, 1977). In this way it might be possible to prevent the formation of biologically hazardous Agrobacteria such as those represented in B and C of Figure 5.1.

6.3. MATERIALS AND METHODS

6.3.1 Bacterial cultures

Bacteria used in conjugations are listed in Table 6.1.

6.3.2 Media and growth conditions

Agrobacterium and *E. coli* strains were grown in LB medium with aeration. *Agrobacterium* strains were incubated at 26^{0}C and *E. coli* strains at 37°C. *Rhizobium* strains were grown in TY medium supplemented with 7 mM $CaCl_2$ at 26°C.

Table 6.1 Bacterial strains used in conjugation experiments

Species	Strain	Plasmid	Origin
Escherichia coli	J53	RP4	Datta *et al.*, 1971
Agrobacterium tumefaciens	C58	pTiC58 Cryptic	M. van Montagu, Ghent
	C58Cl	Cryptic	M. van Montagu, Ghent
	C58rif^R C58nal^R C58ery^R	pTiC58 Cryptic	Spontaneous mutants of C58. This work.
	C58Clrif^R	Cryptic	Spontaneous mutant of C58Cl. This work.
	396::Tn7	pAt396 pTi396 Cryptic	Transposon containing derivative of 396, M. van Montagu, Ghent.

Table 6.1 cont.d. Bacterial strains

Species	Strain	Plasmid	Origin
Rhizobium meliloti	RF6nal^R	2 plasmids, unclassified	Spontaneous mutant of RF6. This work.
	RF10nal^R	2 plasmids, unclassified	Spontaneous mutant of RF10. This work.
	RF14nal^R	1 plasmid, unclassified	Spontaneous mutant of RF14. This work.
	RF22nal^R	1 plasmid, unclassified	Spontaneous mutant of RF22. This work.
Rhizobium leguminosarum	TU1nal^R	1 plasmid, unclassified	Spontaneous mutant of TU1. This work.
Rhizobium lupini	VK10nal^R	1 plasmid, unclassified	Spontaneous mutant of VK10. This work.
Rhizobium japonicum	WB1 nal^R	1 plasmid, unclassified	Spontaneous mutant of WB1. This work.
Rhizobium trifolii	SR4 nal^R	2 plasmids, unclassified	Spontaneous mutant of SR4. This work.
	SQ9 nal^R	1 plasmid, unclassified	Spontaneous mutant of SQ9. This work.
	SACL nal^R	1 plasmid, unclassified	Spontaneous mutant of SACL. This work.

All *Rhizobium* strains were obtained from the Department of Plant Protection, South Africa.

6.3.3 Antibacterial drugs

Antibacterial drugs were added to the media at the following final concentrations (µg/ml): rifampicin 25; erythromycin 25; naladixic acid 100; kanamycin 50; streptomycin 100; spectinomycin 100.

6.3.4 Selection for antibiotic resistant bacteria

Strains (listed in Table 6.1) were grown overnight in the appropriate media at 26°C. Twenty millilitres of LA or TY agar containing the appropriate concentration of antibiotic (as in section 6.3.3) were poured into petri plates. The plates were slanted such that a slope of agar was formed. Twenty millilitres of LA or TY agar without antibiotic were poured on to the solidified slope of agar. The agar was allowed to set. Overnight cultures were concentrated ten times and 0.1 ml plated on to the correct antibiotic gradient plates. The plates were incubated for four days. Colonies appearing on the high concentration region of the plate were picked and purified.

6.3.5 Conjugation

The conjugations carried out are listed in Table 6.2. Donor and recipient bacteria were grown to log phase

in LB at the appropriate temperature. One millilitre each of donor and recipient bacteria were mixed together with 2 mls of LB. Aliquots (200 µl) were spotted onto the centre of an LA plate. The plates were incubated overnight at 26°C. For conjugations with the Rhizobium strains, 0.2 mls of the recipient were spotted on to TY plates and grown overnight at 26°C before addition of 0.2 mls of the donor strain. Donor and recipient were incubated at 26°C for 72 hrs. Ater incubation, the bacteria were resuspended in 1 ml of saline (0.8% NaCl) and plated on selective plates to determine the number of donor, recipient and transconjugant bacteria. Transconjugant bacteria carrying RP4 and Tn7 were selected on 50 µg/ml of kanamycin and 100 µg/ml of streptomycin and spectinomycin, respectively.

6.3.6 Assay for agrocin production

Transconjugant bacteria carrying RP4 and Tn7 were assayed for the production of agrocin according to the methods of Kerr and Htay(1974) and Mayr-Harting et al., (1972)(see section 2.3.2).

6.3.7 Screening for plasmids in transconjugants

Transconjugant strains were screened for the presence of plasmids using the method of Kado and

Liu(1981)(see section 3.3.8). In order to obtain the optimum conditions for the extraction of plasmids in each strain, bacteria were treated with heat for different lengths of time. The plasmids were scanned as in section 3.3.8.

6.3.8 Determination of the ability of agrocin producing Rhizobium transconjugants in preventing gall formation

Tests were carried out on potato slices as in section 3.3.10. Overnight cultures of Rhizobium strains, agrocin producing Rhizobium transconjugants, C58, and mixtures of Rhizobium and C58 strains were inoculated on to potato slices, and incubated for 14 days at 26°C.

6.4 RESULTS

One way of trying to extend biological control of crown gall is by transferring the genes encoding agrocins to benign bacterial soil species which can be used to coat seedlings, roots or shoots before planting. It can be biologically hazardous to use *Agrobacterium* strains in biological control due to Ti plasmid transfer especially if the ratio of pathogen to non-pathogen is more than one. Hence, an attempt was made to transfer the agrocin 84 encoding plasmid, pAt396, to *Rhizobium* species using the plasmid RP4 which is known to have mobilising ability(Holloway, 1979).

In order to perform the necessary conjugations, drug resistant mutants of potential recipients had to be isolated. Mutants readily appeared in the region of the gradient plates containing a high concentration of antibiotic. The mutants were stable in the presence of the respective antibiotics. Mutant strains had a slower growth rate than their parental strains. Stationary phase cultures of antibiotic resistant *A. tumefaciens* and *Rhizobium* strains was achieved within 48 and 96 hrs respectively at 26^{0}C, whereas a culture of the same density was achieved within 24 and 48 hrs respectively by the parental strains. The colony morphology of the mutants were

the same as that of the parental strains.

A number of conjugation steps were carried out in order to determine whether RP4 could form an RP4::pAt396 cointegrate with pAt396. The incentive for wanting to obtain a cointegrate was that transfer of pAt396 to Rhizobium would be more efficient than mobilisation by RP4 as a separate entity.

6.4.1 Cross J53(RP4) x 396::Tn7(pAt396)

Plasmid RP4 was transferred from E. coli J53(RP4) to A. tumefaciens 396::Tn7 which carries the agrocin encoding plasmid, pAt396. Donors, recipients and transconjugants were selected on plates containing the relevant antibiotics. The frequency of transfer of RP4 was not determined but individual colonies were assayed for the production of agrocin 84. All transconjugants produced agrocin. This indicated that all were 396::Tn7 transconjugants carrying RP4 and the genes for agrocin production (pAt396) (Table 6.2).

Examination of the plasmid profiles as well as the spectrophotometric scans of the gels of the donor, recipient and transconjugants showed that (i) plasmid RP4 was present in the donor strain J53(RP4) (Figures 6.2a and b) (ii) the recipient strain,

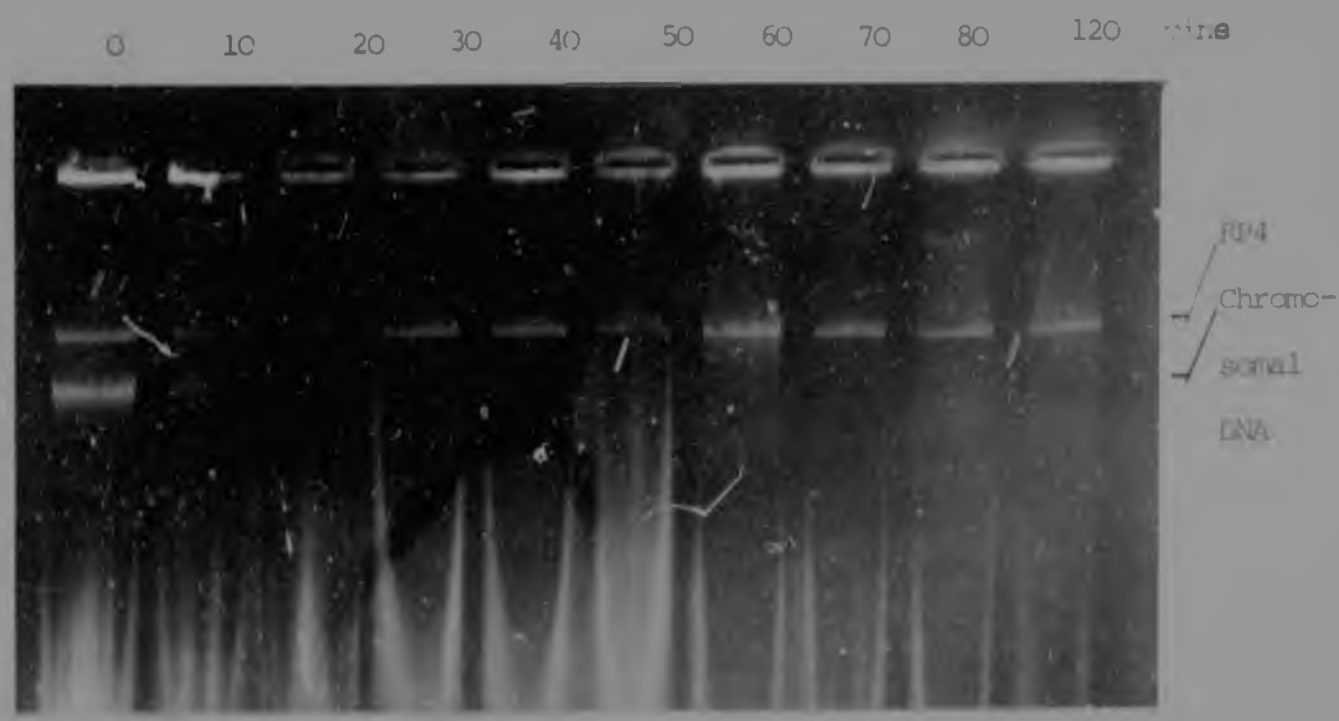


Figure 6.2a Plasmid profile of J53(RP4). Plasmids were extracted according to the method of Kado and Liu(1981). Bacteria were heat treated for various lengths of time to extract the plasmids (55°C).

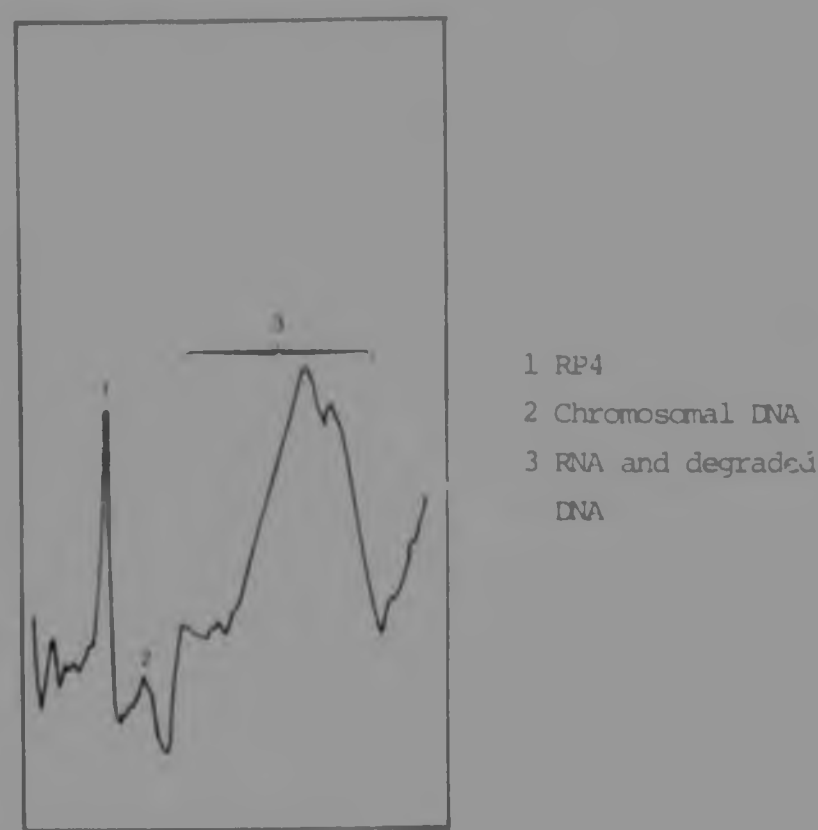


Figure 6.2b Spectrophotometric scan of the plasmids of J53(RP4) extracted at 55°C for 40 min (lane 5).

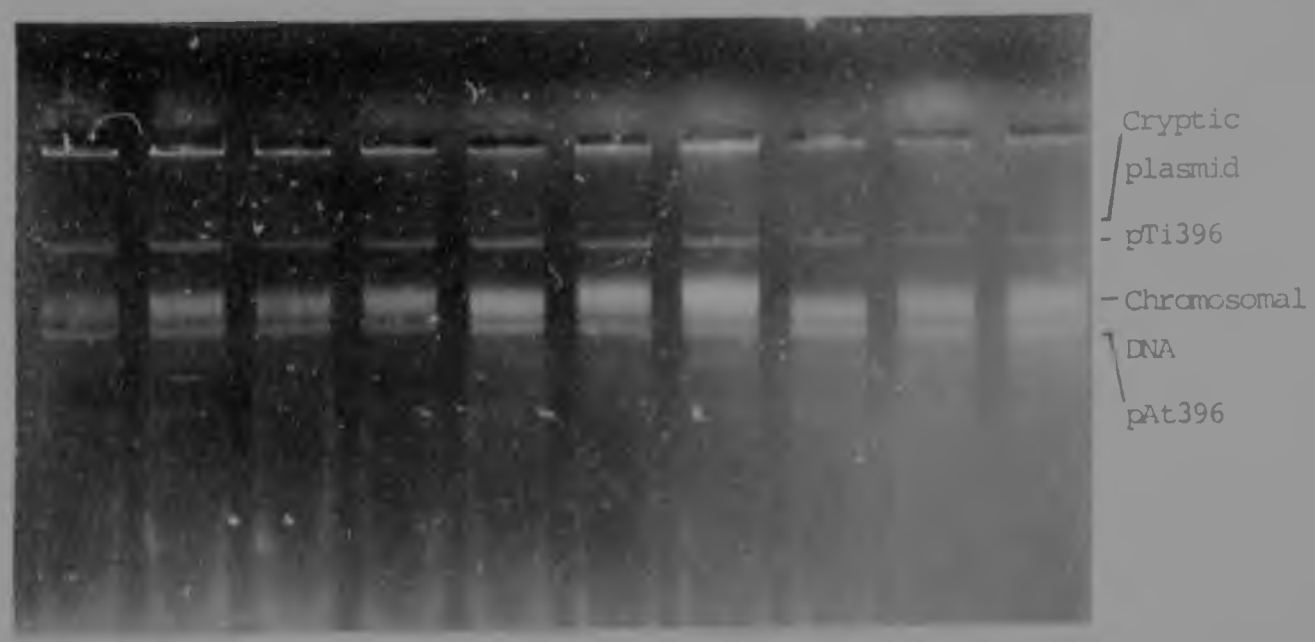


Figure 6.3a Plasmid profile of 396::Tn7. Plasmids were extracted at 95°C for various lengths of time

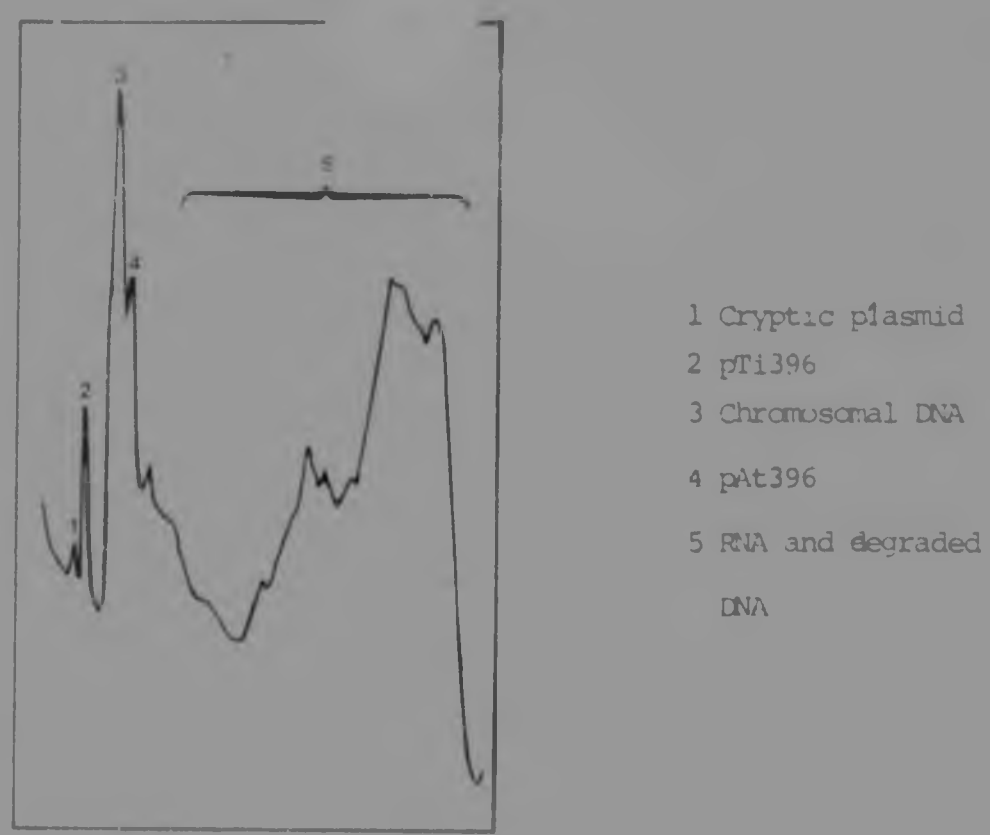


Figure 6.3b Spectrophotometric scan of the plasmids of 396::Tn7 extracted at 95°C for 7 min (lane 5).

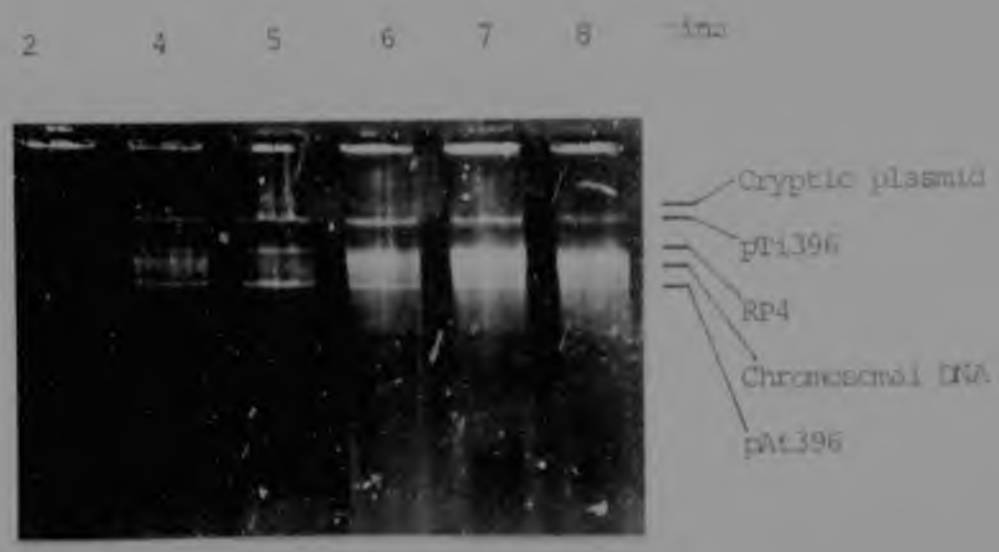
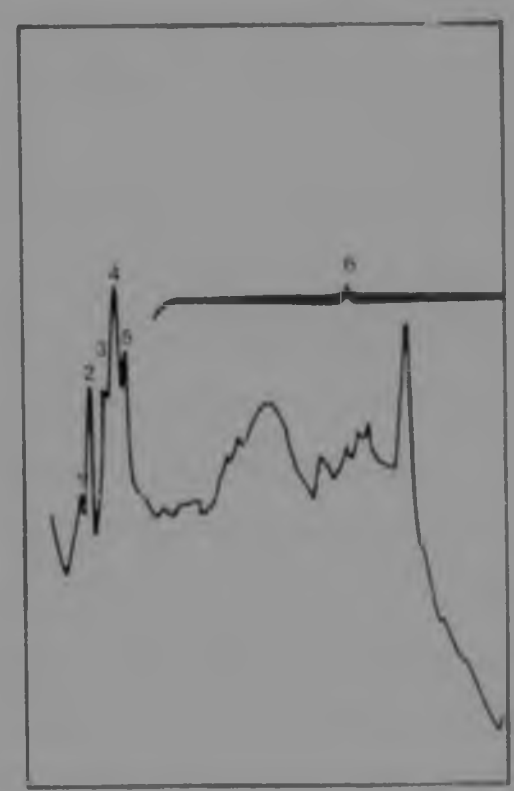


Figure 6.4a Plasmid profile of 396::Tn7(RP4). Plasmids were extracted at 95°C for various lengths of time.



1 Cryptic plasmid
2 pTi396
3 RP4
4 Chromosomal DNA
5 pAt396
6 RNA and degraded DNA

Figure 6.4b Spectrophotometric scan of the plasmids of 396::Tn7(RP4) extracted at 95°C for 4 min (lane 2).

396::Tn7(pAt396) harboured three plasmids, the cryptic plasmid, the Ti plasmid, pTi, and the agrocin encoding plasmid pAt396 (Figure 6.3a and b), and (iii) the transconjugant 396::Tn7(RP4)(pAt396) harboured the plasmids of both donor and recipient strains (Figures 6.4a and b).

6.4.2 Cross 396::Tn7(RP4)(pAt396) x C58rif^R or C58Clrif^R

As it has been shown for plasmid R68.45, a derivative of RP4, that not all strains harbouring the plasmid have efficient mobilising ability(Holloway, 1979), five agrocin producing transconjugant colonies were used as donors of RP4 and pAt396 in a second round of conjugation to C58rif^R and C58Clrif^R. Both crosses were carried out as it was not known whether the presence of the nopaline plasmid which encodes sensitivity to agrocin 8. would affect agrocin plasmid transfer and expression. The frequencies of transfer of RP4 and Tn7 are given in Table 6.2. RP4 was transferred at a rate of 10^{-4} per donor and recipient, and Tn7 was transferred at a rate of 10^{-5} per donor and recipient. The inheritance of Tn7 was of interest as strain 396::Tn7, a derivative of 396, produced higher levels of agrocin than the parental strain. All colonies growing on kanamycin were patched on to streptomycin and spectinomycin plates

and vice versa. It was found that all colonies which grew on streptomycin and spectinomycin also grew on kanamycin plates and that only 10% of the colonies growing on kanamycin grew on streptomycin and spectinomycin. Therefore the transfer of RP4 was essential for the transfer of Tn7. Transconjugant colonies carrying RP4, and RP4 as well as Tn7 were assayed for the production of agrocin (Table 6.2). There was 1.6% transfer and expression of the agrocin encoding plasmid in C58rif^R transconjugants carrying RP4 and Tn7 and 2.0% transfer to C58Clrif^R transconjugants. Transfer and expression of the agrocin plasmid in C58rif^R transconjugants carrying RP4 only occurred at a rate of 1.7% compared to the 2.6% rate of transfer to C58Clrif^R transconjugants. It therefore appeared as though neither the Ti plasmid nor Tn7 had an effect on the transfer and expression of the agrocin plasmid. The levels of agrocin produced by Tn7 carrying transconjugants were not higher than those produced by transconjugants carrying only RP4. Therefore the mere presence of Tn7 is not sufficient to affect agrocin production. It would be interesting to determine where Tn7 is integrated in the transposon derivative of 396 to allow it to produce higher levels of agrocin.

The plasmid profiles and the spectrophotometric scans of the plasmids of recipient strains C58rif^R and

C58Clrif^R are shown in Figures 6.5a and b and 6.6a and b, respectively. Spectrophotometric scans and plasmid profiles of C58rif^R::Tn7(RP4)(pAt396) and C58Clrif^R::Tn7(RP4)(pAt396) transconjugants are shown in Figures 6.7a and b and 6.8a and b, respectively. C58rif^R was shown to harbour a cryptic as well as a Ti plasmid, whereas the transconjugant was found to harbour a cryptic plasmid, a Ti plasmid, RP4 and pAt396. The same was observed for C58Clrif^R and C58Clrif^R::Tn7(RP4)(pAt396), except that a Ti plasmid was absent in these two strains.

6.4.3 Cross C58rif^R::Tn7(RP4)(pAt396) x C58nal^R and C58Clrif^R::Tn7(RP4)(pAt396) x C58nal^R

In order to determine whether the C58 and C58Cl transconjugants as shown above, which carried both RP4 and pAt396 could allow the formation of a cointegrate plasmid which would then co-transfer both sets of markers at equal frequency, five exconjugants (3 C58rif^R::Tn7(RP4)(pAt396) and 2 C58Clrif^R ::Tn7(RP4)(pAt396)) were crossed with a C58nal^R recipient. The frequency of transfer of RP4 and Tn7 was 10^{-1} per donor and per recipient, and transfer of all markers was linked i.e. one hundred percent co-transfer of Tn7 and RP4 was observed. This indicated the probable transposition of Tn7 on to RP4. Two hundred transconjugants from each of the

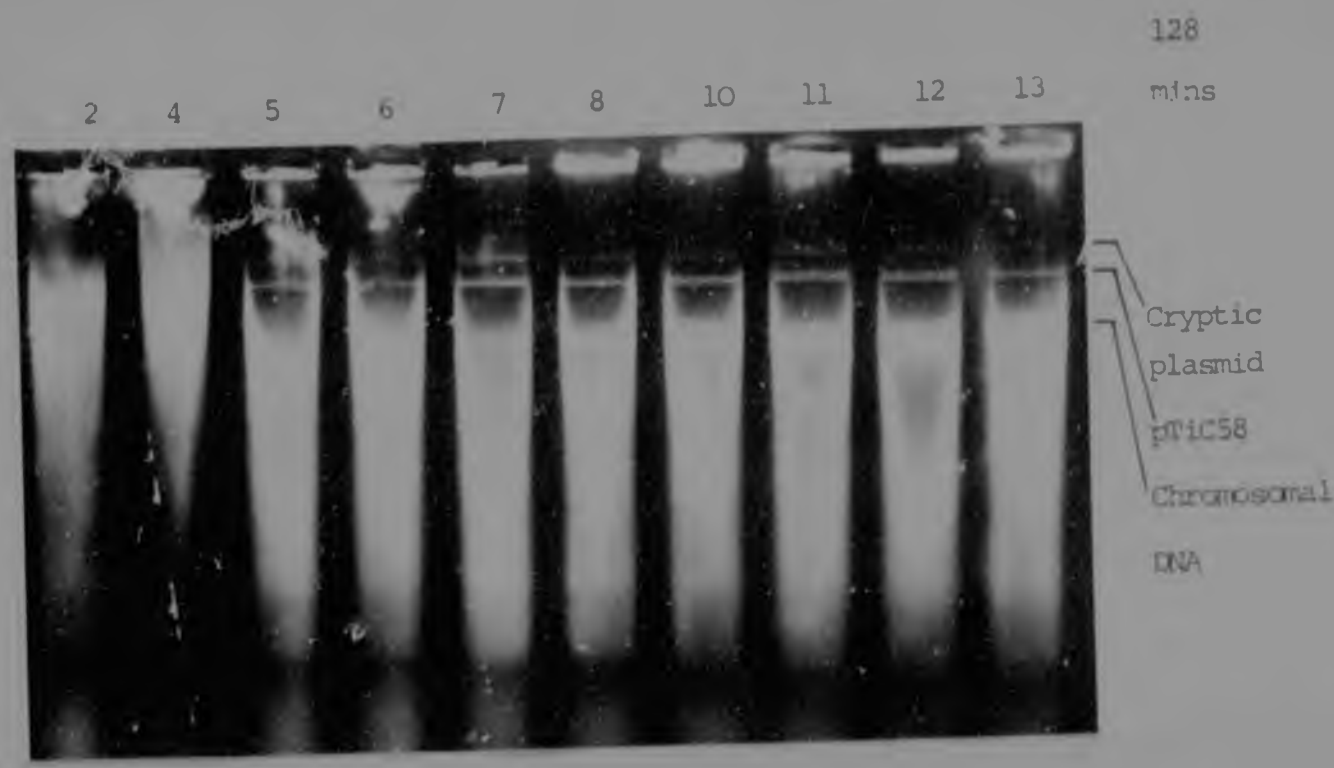


Figure 6.5a Plasmid profile of C58rif^{R}. Plasmids were extracted at 95°C for various lengths of time.

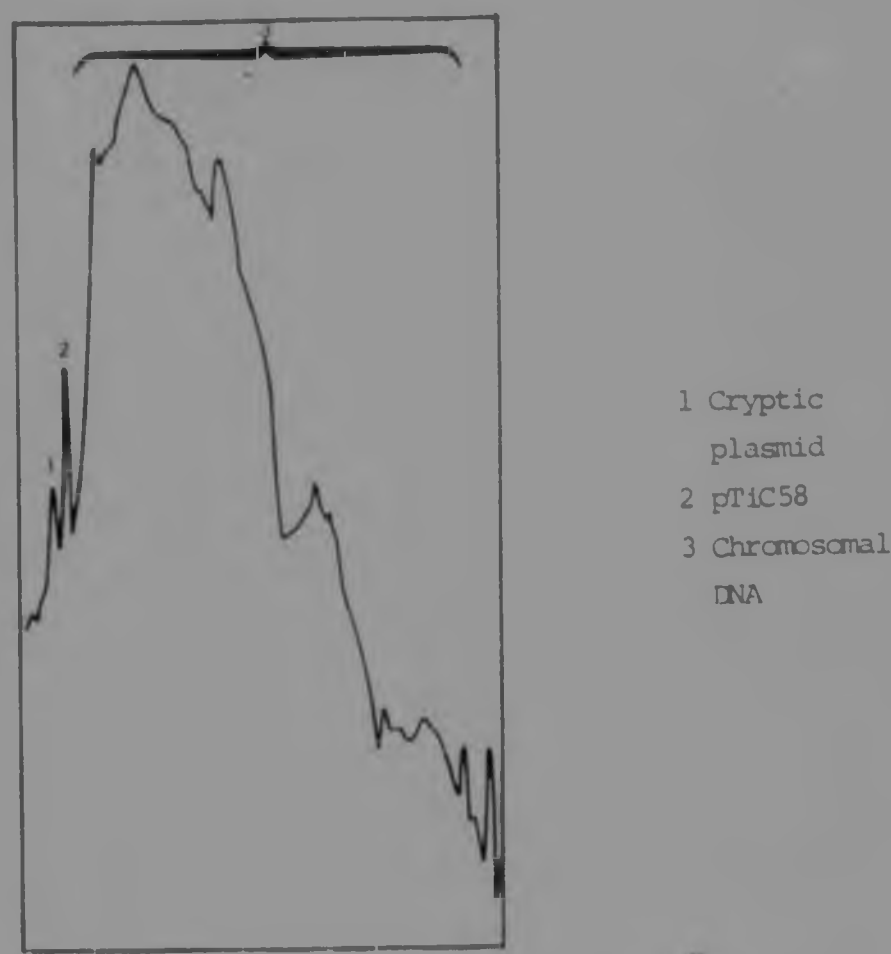


Figure 6.5b Spectrophotometric scan of the plasmids of C58rif^{R} extracted at 95°C for 7 min (lane 5).

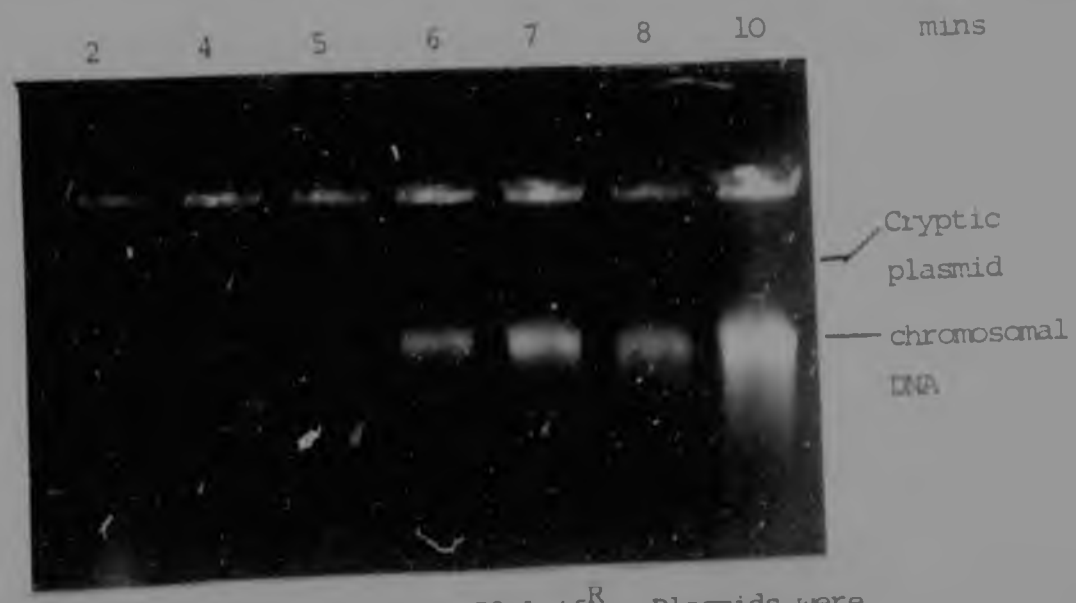


Figure 6.6a Plasmid profile of C58Clrif^{R}. Plasmids were extracted for various lengths of time at 95°C.

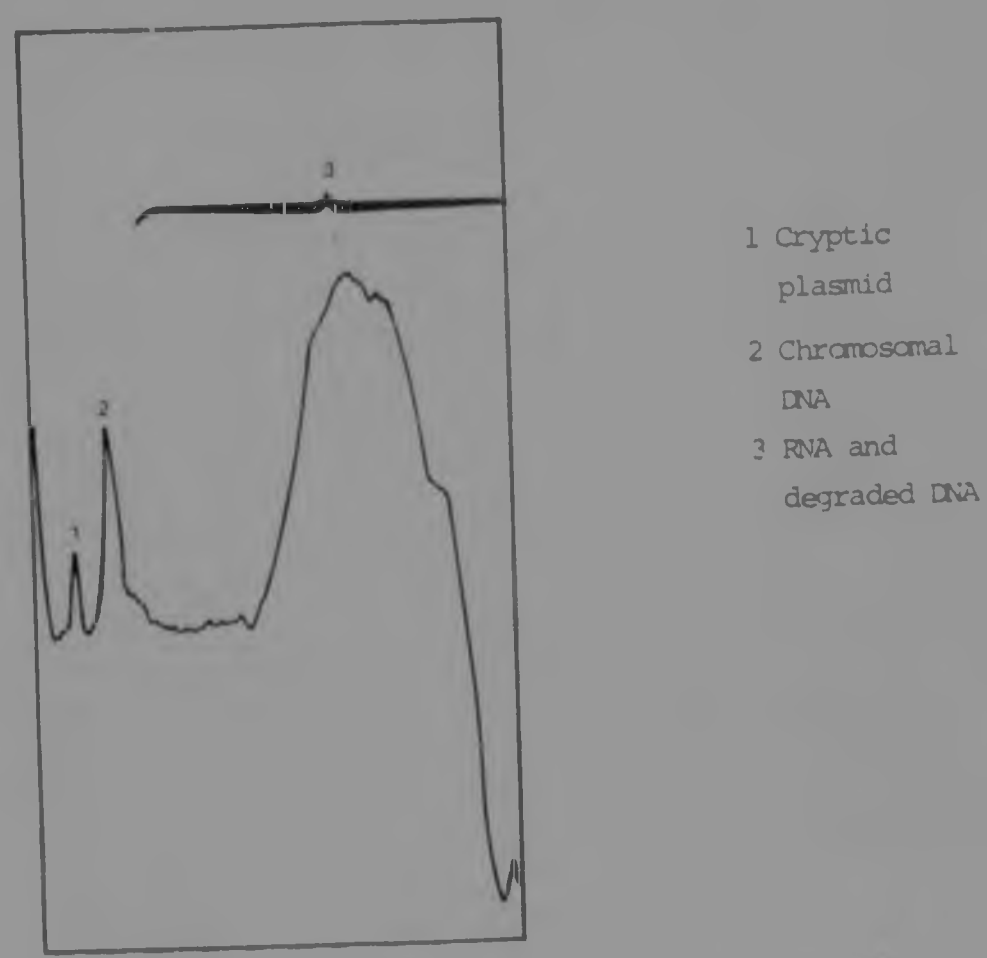


Figure 6.6b Spectrophotometric scan of the plasmids of C58Clrif^{R} extracted at 95°C for 5 min (lane 3).

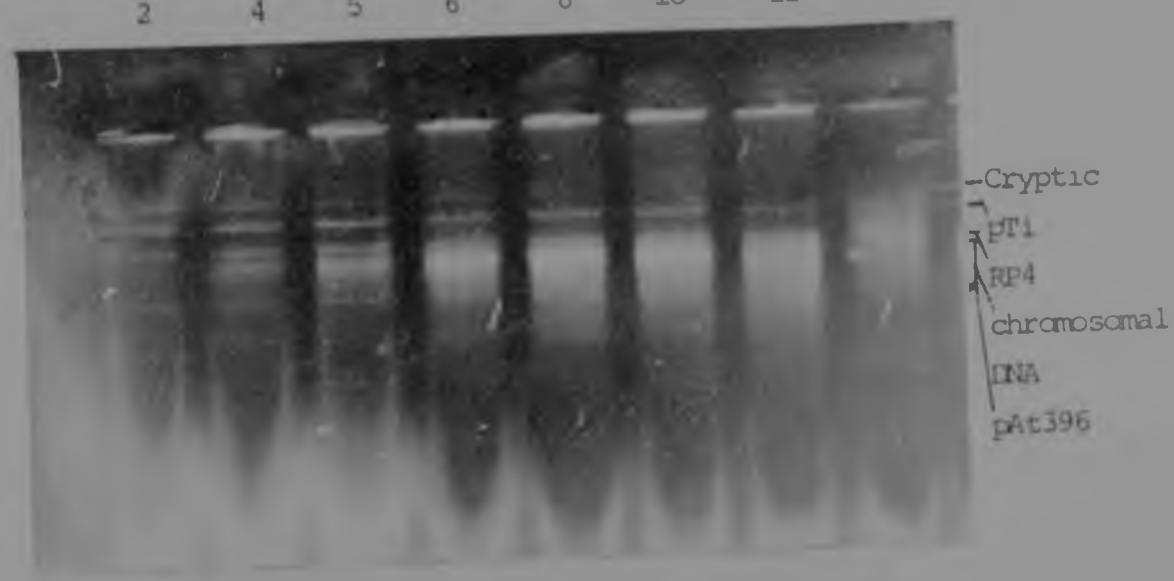
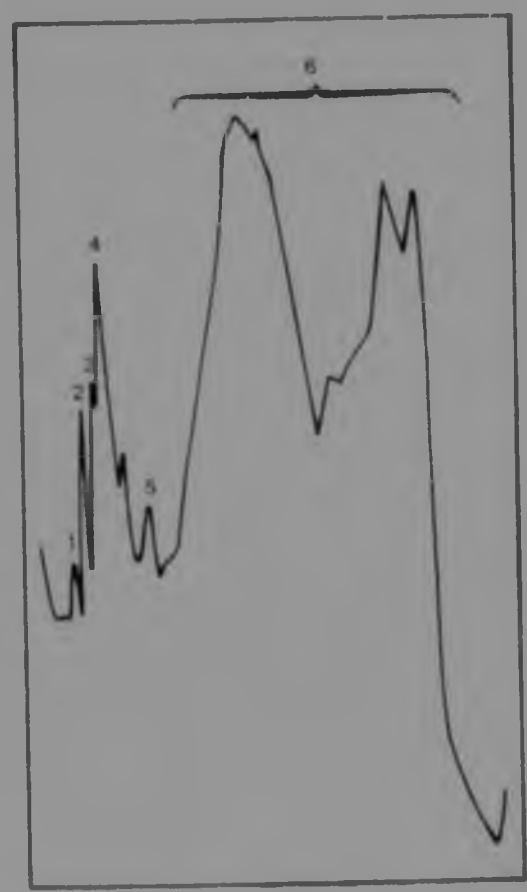


Figure 6.7a Plasmid profile of C58rif^{R}::Tn7(RP4)(pAt396). Plasmids were extracted at 95°C for various lengths of time.



1 Cryptic plasmid
2 pTi
3 RP4
4 chromosomal DNA
5 pAt396
6 RNA and degraded DNA

Figure 6.7b Spectrophotometric scan of the plasmids of C58rif^{R}::Tn7(RP4)(pAt396) extracted at 95°C for 4 min.

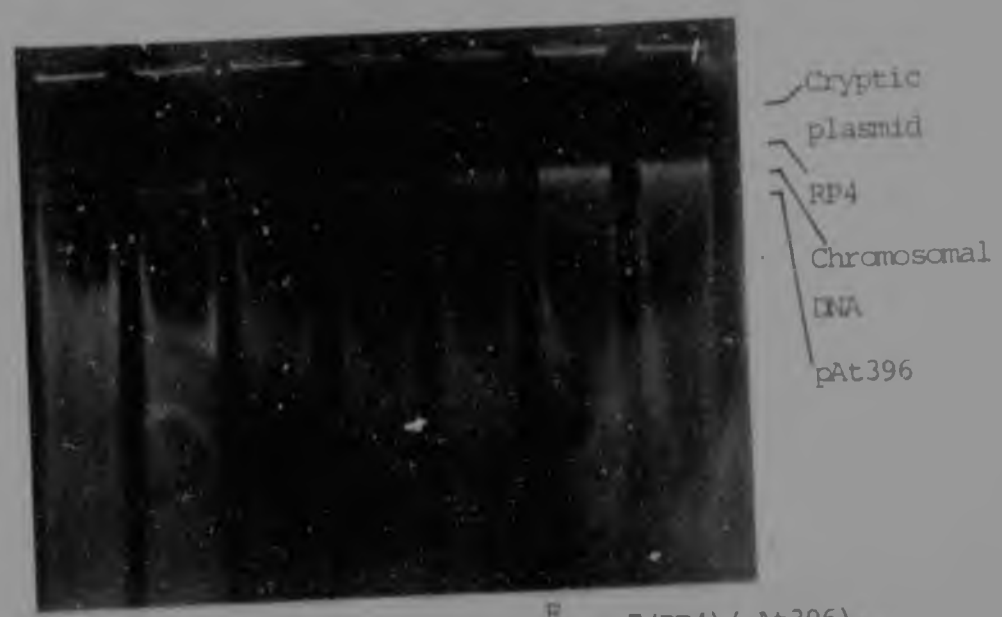


Figure 6.8a Plasmid profile of C58Clrif^{R}::Tn7(RP4)(pAt396). Plasmids were extracted for various lengths of time at 95°C.

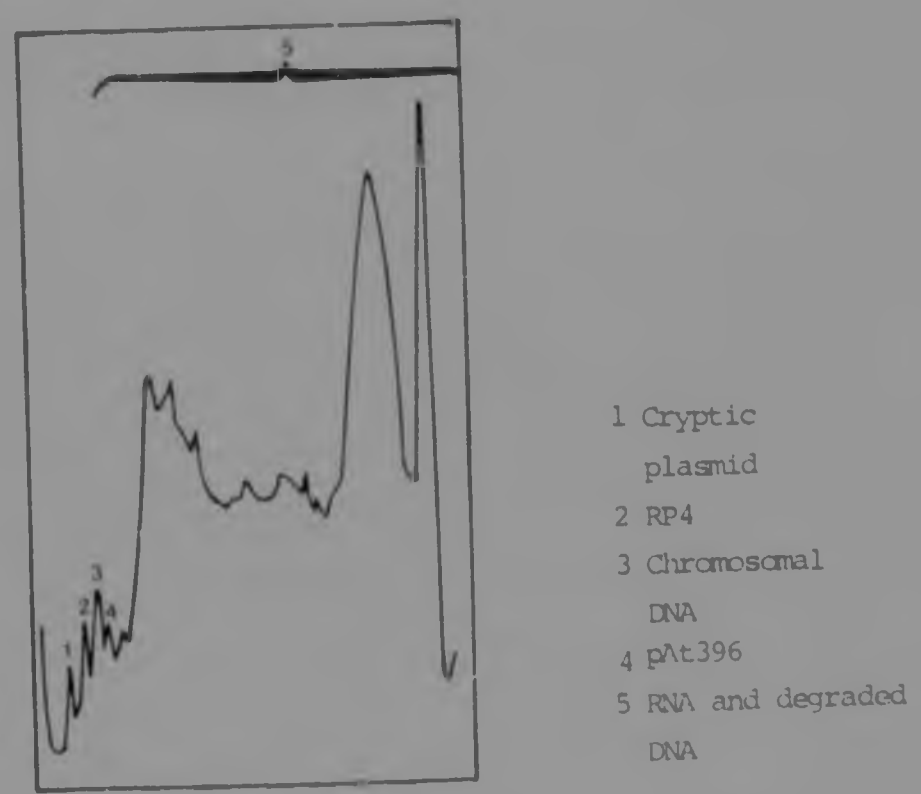


Figure 6.8b Spectrophotometric scan of the plasmids of C58Clrif^{R}::Tn7 (RP4)(pAt396) extracted at 95°C for 2 min (lane 1).

five crosses were assayed for agrocin. The results are shown in Table 6.2. In four of the five crosses, only 2.2% to 11% of the RP4 transconjugants also inherited pAt396, while in one, all the transconjugants co-inherited pAt396. The plasmid profile and the spectrophotometric scan of a C58nal^R ::Tn$^-$(RP4)(pAt396) transconjugant is shown in Figure 6.9a and b. This strain is one of the transconjugants from the cross showing 100% co-transfer of RP4 and pAt396 i.e. using donor number 3. It was found to harbour a cryptic plasmid, a Ti plasmid, RP4 and pAt396, showing that a stable RP4::pAt396 cointegrate was not present.

6.4.4 Cross C58nal^R ::Tn7(RP4)(pAt396) x C58ery^R

It was originally thought that the formation of an RP4::pAt396 plasmid cointegrate would be advantagous for the transfer of pAt396 between strains. The plasmid screens of donor number 3 (Figure 6.7a and b) which conferred on recipients 100% co-inheritance of all the markers showed that a stable cointegrate was not present. With this knowledge it was important to determine the general efficiency of mobilisaton of pAt396 by RP4. To this end, four nal^R transconjugants which had resulted from the cross using donor number 3 (100% co-transfer) were used as donors in a subsequent cross with C58ery^R .

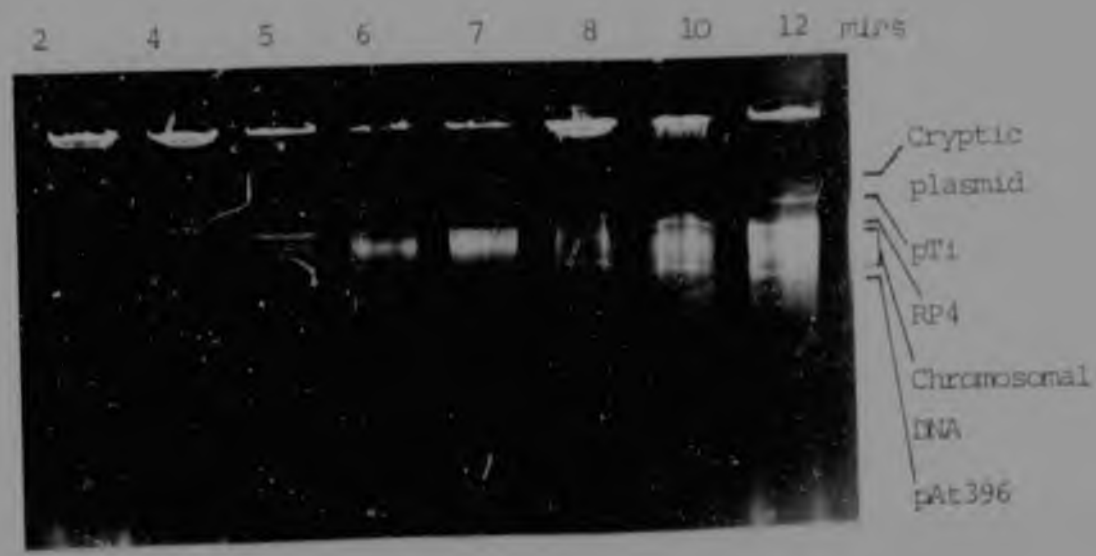
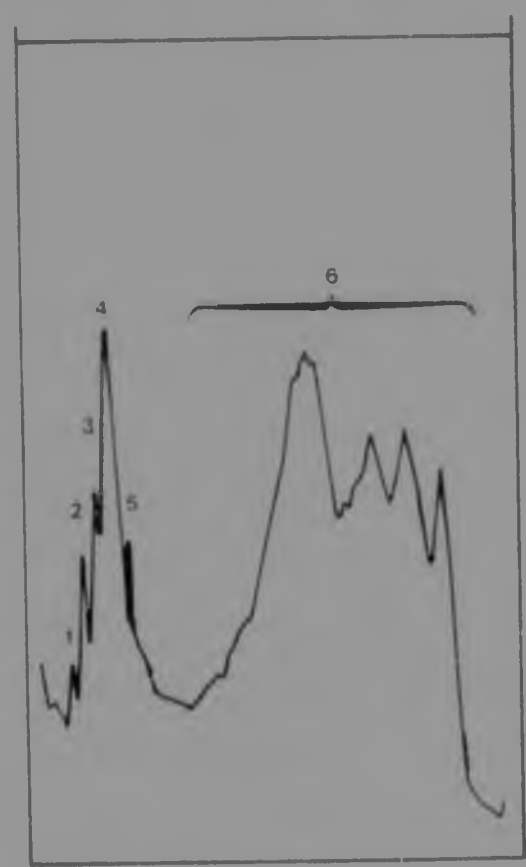


Figure 6.9a. Plasmid profile of C58nal^{R}::Tn7(RP4)(pAt396). Plasmids were extracted at 95°C for various lengths of time.



1. Cryptic plasmid
2 pTi
3 RP4
4 Chromosomal DNA
5 pAt396
6 RNA and degraded DNA

Figure 6.9b. Spectrophotometric scan of the plasmids of C58nal^{R}::Tn7(RP4,(pAt396) extracted at 95°C 5 min (Lane 3)

Plasmid RP4 and Tn7 were co-transferred at a rate of 10^{-2} per donor and per recipient (Table 6.2). Two hundred transconjugants from four separate crosses were screened for the production of agrocin, but co-inheritance of RP4 and pAt396 varied from 0 to 11% in the four crosses (Table 6.2). This indicated that although a cointegrate plasmid did not exist in any of the donor strains, mobilisation of pAt396 by RP4 occurred at detectable frequencies.

6.4.5 Cross C58rif^R::Tn7(RP4)(pAt396) x 10 different *Rhizobium* strains

Although a cointegrate RP4::pAt396 plasmid had not been formed, the C58rif^R::Tn7(RP4)(pAt396) donor number 3 (in which pAt396 was mobilised with RP4 to C58nal^R in 100% of the transconjugants tested) was used to mobilise pAt396 to different species of *Rhizobium*. The plasmid profiles of the *Rhizobium* recipients are shown in Figure 6.10. RP4 was transferred to all *Rhizobium* strains at different frequencies (Table 6.3) ranging from 1×10^{-4} to 1.75×10^{-6} per recipient. Five hundred transconjugants from each cross were tested for the ability to produce agrocin. Only seven of them were found to produce agrocin. Four of the transconjugants resulted from the cross using *R. meliloti* RF10nal^R as recipient, two

Figure 6.10 Plasmid profiles of nal^R Rhizobium strains extracted at 65°C for 0 min (lane 1), 10 min (lane 2), and 20 min (lane 3). a) RF6, b) RF10, c) RF14, d) RF22, e) TU1, f) VK10, g) WB1 h) SR4, i) SQ9, and j) SAC1.

using R. meliloti RF6nal^R as recipient, and one using R. leguminosarum TU1nal^R . To verify that these were not spontaneous rif^R donors, transconjugants were checked and found to be rif^S .

Figure 6.11a and 6.12a show the plasmid profiles of the two R. meliloti RF6 transconjugants. RF6nal^R transconjugant number 1 (Figure 6.11) was found to harbor four plasmids. The two largest plasmids were the same size as those found in the recipient RF6nal^R. The plasmid RP4 [illegible] also found in the transconjugant. pAt396 was not [illegible]ent. However, a plasmid with higher mobility than pAt396 was observed. This plasmid ma[illegible]ncode agrocin production.

Only three plasmids were observed in RF6nal^R transconjugant number 2. Two of these were derived from the recipient RF6nal^R . The third plasmid was the same size as RP4 found in the donor C58rif^R ::Tn7(RP4)(pAt396). No plasmid similar in size to pAt396 was found in this transconjugant.

Three of the four R. meliloti RF10nal^R transconjugants (numbers 1, 2 and 3; Figures 13 and 14) appeared to harbour the two plasmids of the recipient RF10nal^R , as well as RP4, which was received from the donor. A plasmid which was smaller in size than pAt396 was also observed, and could

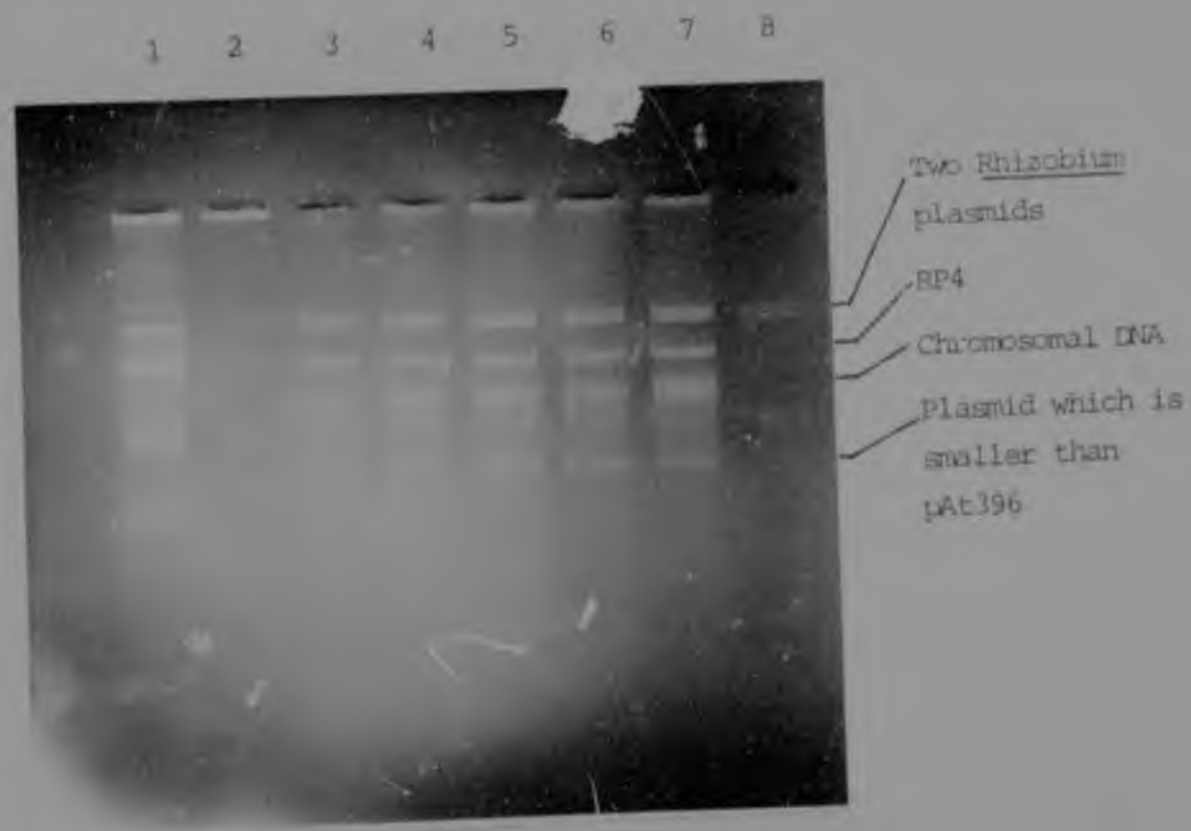


Figure 6.11a Plasmid profile of C58rif^R::Tn7(RP4)(pAt396) (lane 1), RF6nal^R transconjugant number 1 extracted at 65°C for 0 min (lane 2), 2 min (lane 3), 4 min (lane 4), 6 min (lane 5), 8 min (lane 6), 10 min (lane 7), RF6nal^R recipient (lane 8).

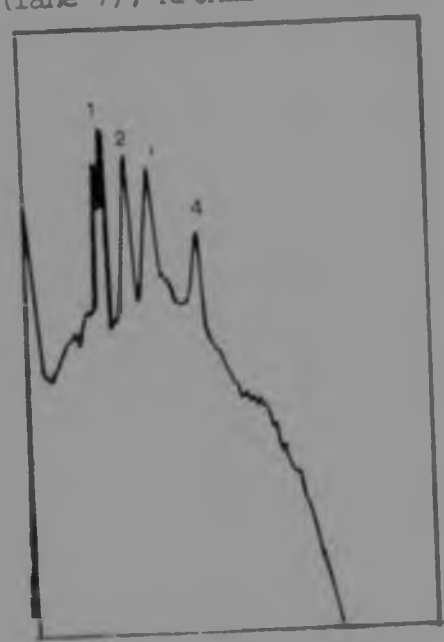


Figure 6.11b Spectrophotometric scan of the plasmids of RF6nal^R transconjugant number 1.

1 Two Rhizobium plasmids
2 RP4
3 Chromosomal DNA
4 Plasmid which is smaller than pAt396.

Figure 6.11c Spectrophotometric scan of the plasmids of RF6nal^R recipient.

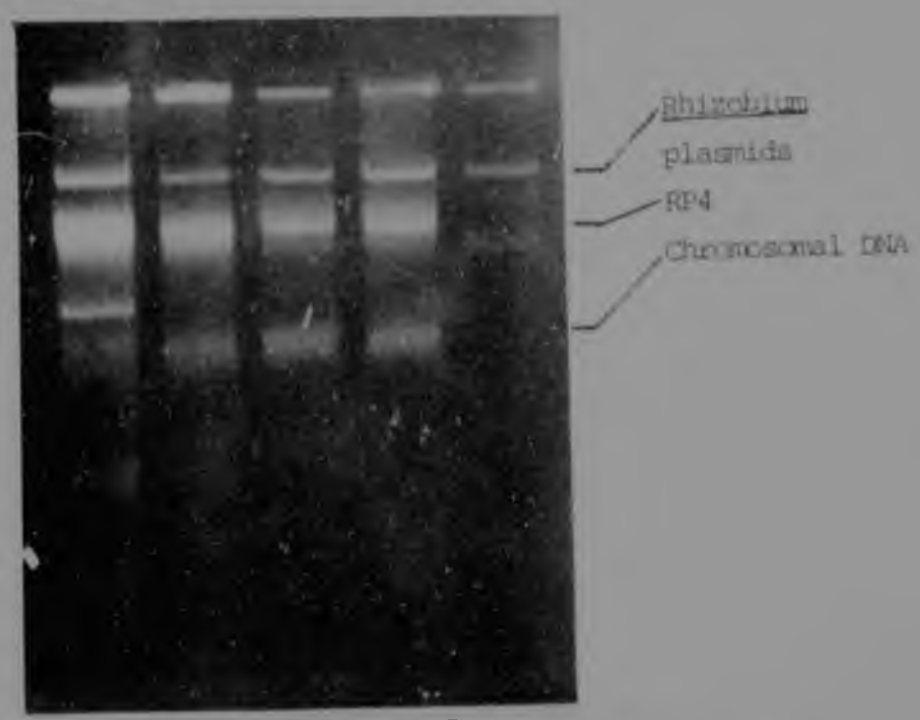


Figure 6.12a Plasmid profile of C58rif^{R}::Tn7(RP4)(pAt396) (lane 1), RF6nal^{R} transconjugant number 2 extracted at 65°C for 0 min (lane 2), 5 min (lane 3), 10 min (lane 4), and RF6nal^{R} recipient (lane 5).

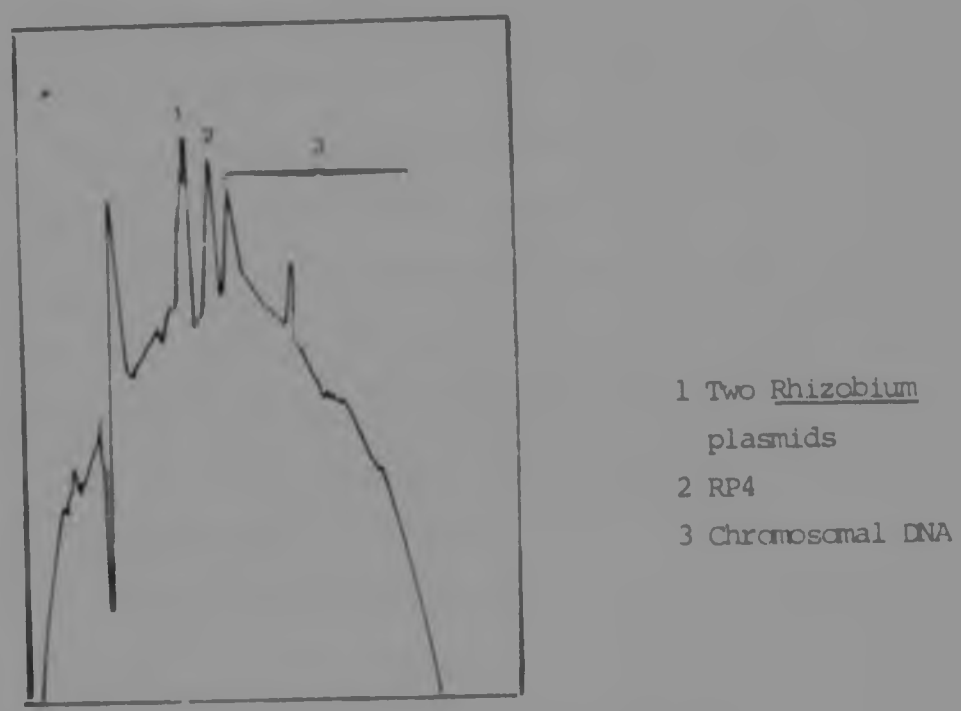


Figure 6.12b Spectrophotometric scan of the plasmids of RF6nal^{R} transconjugant number 2.

encode agrocin production.

One of the RF10nal^R transconjugants (number 4; Figure 6.14) had the same profile as the donor C58rif^R ::Tn7(RP4)(pAt396). The plasmids in the R. leguminosarum TU1nal^R transconjugant also resembled those of the donor. These two transconjugants harboured four plasmids, similar in size to the cryptic plasmid, the Ti plasmid, RP4 and pAt396 of the donor. This result indicated either that these strains were in fact donor strains which had simultaneously lost their rif^R marker and mutated to nal^R, or that they were recipients which had received all the plasmids of the donor strain. Tests were carried out to investigate which of these had occurred. Tests on potato slices showed that the R. meliloti RF10nal^R transconjugant number 4 and the R. leguminosarum TU1nal^R transconjugant were in fact donors which had reverted to rif^S and spontaneously became nal^R. These two transconjugants produced galls on potato slices. The five remaining R. meliloti transconjugants (transconjugant RF6nal^R number 1 and 2 and RF10nal^R numbers 1, 2 and 3) did not induce galls on potato slices. A further test to distinguish between Rhizobium and Agrobacterium was by growth on a medium which selects for A. tumefaciens(Clark, 1969). The medium contains lactose and manganous ions, and selectively grows A.

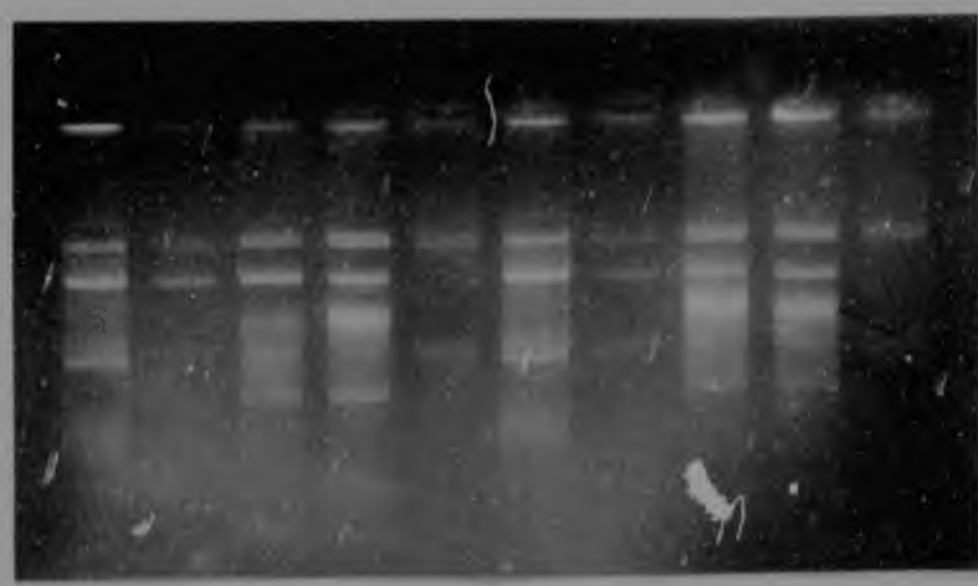


Figure 6.13a Plasmid profiles of C58rif^{R}::Tn7(RP4)(pAt396) (lanes 1 and 6), RF1Onal^{R} transconjugant number 1 extracted at 65°C for 0 min (lane 2), 5 min (lane 3), 10 min (lane 4), RF1Onal^{R} recipient (lanes 5 and 10), RF1Onal^{R} transconjugant number 2 extracted at 65°C for 0 min (lane 7), 5 min (lane 8) and 10 min (lane 9).

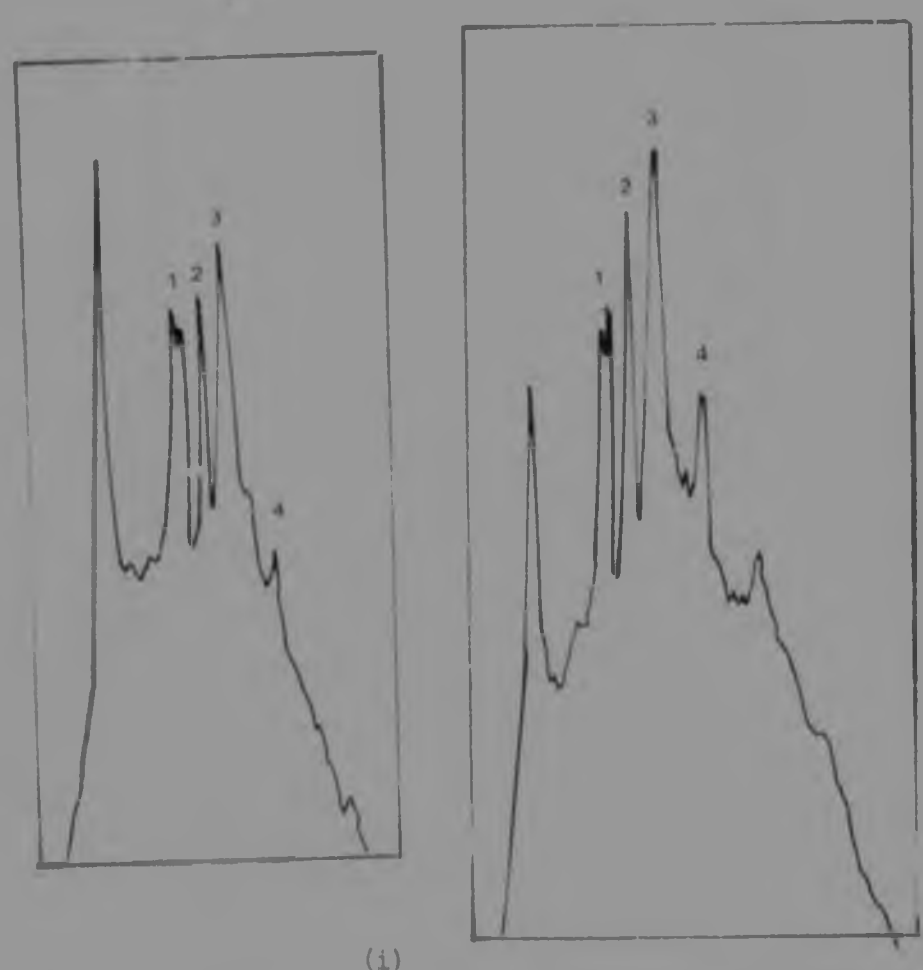


Figure 6.13b Spectrophotometric scan of the plasmids of RF1Onal^{R} transconjugant number 1 (i) and number 2 (ii).

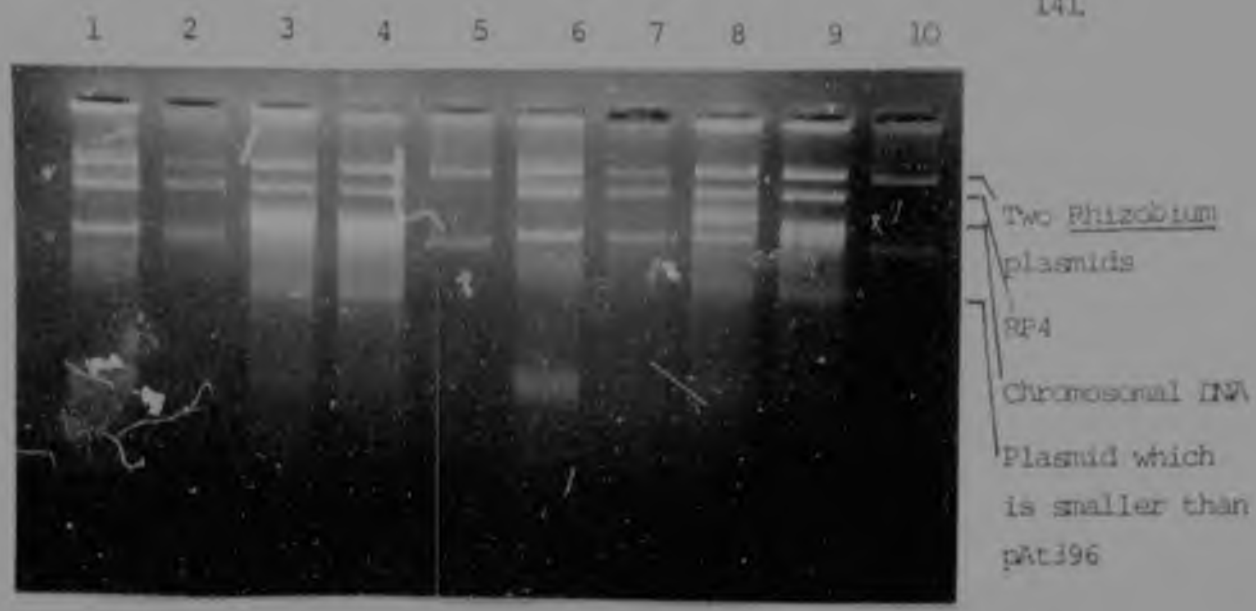


Figure 6.14a Plasmid profile of C58rif^{R}::Tn7(RP4)(pAt396) (lanes 1 and 6), RF1Onal^{R} transconjugant number 3 extracted at 65°C for 0 min (lane 2), 5 min (lane 3), 10 min (lane 4), RF1Onal^{R} recipient (lanes 5 and 10), RF1Onal^{R} transconjugant number 4 extracted at 65°C for 0 min (lane 7), 5 min (lane 8) and 10 min (lane 9).

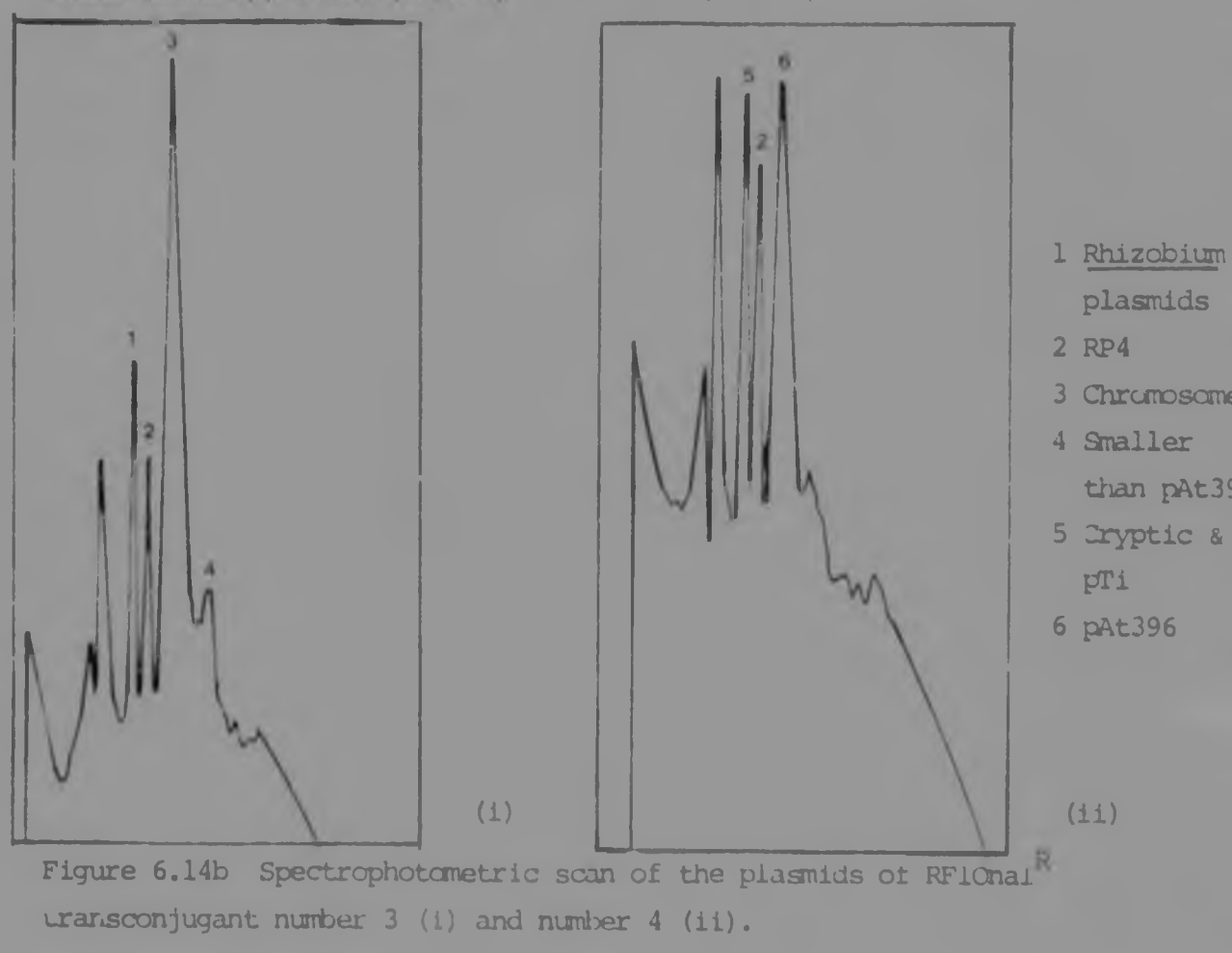


Figure 6.14b Spectrophotometric scan of the plasmids of RF1Onal^{R} transconjugant number 3 (i) and number 4 (ii).

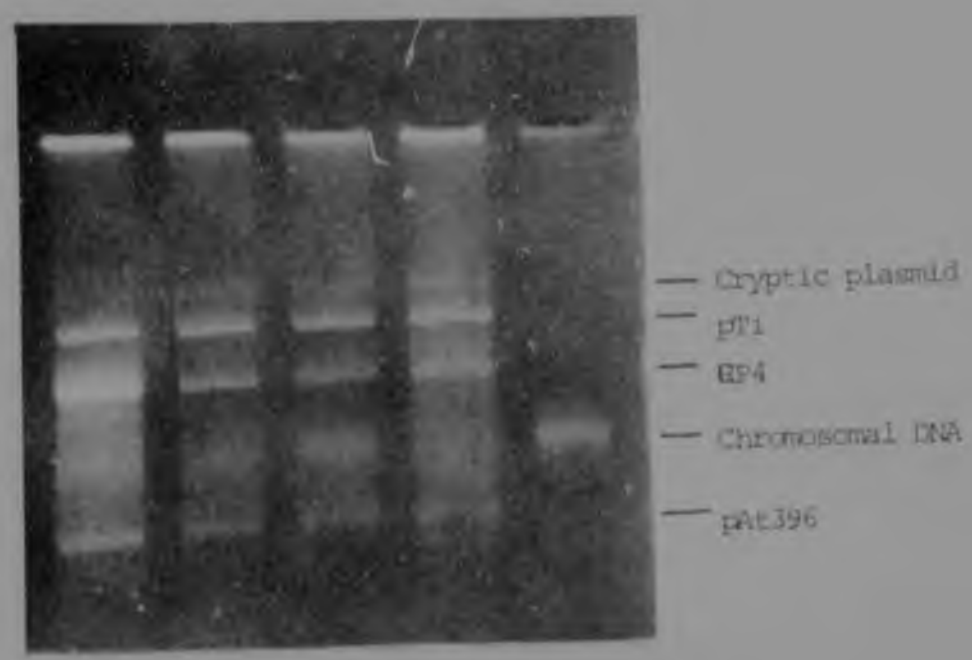
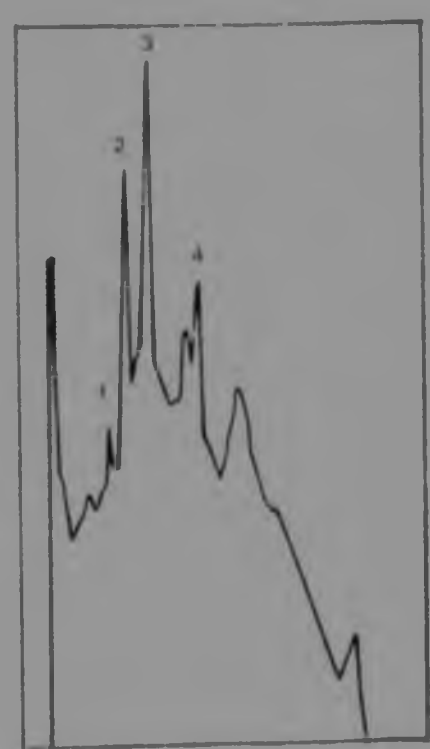


Figure 6.15a Plasmid profile of C58rif^{R}::Tn7(RP4)(pAt396) (lane 1), TUlnal^{R} transconjugant extracted at 65°C for 0 min (lane 2), 5 min (lane 3), 10 min (lane 4) and TUlnal^{R} recipient (lane 5).



1 Cryptic plasmid
2 pTi
3 RP4
4 pAt396

Figure 6.15b Spectrophotometric scan of the plasmids of TUlnal^{R} transconjugant. The scan resembles that of the donor C58rif^{R}::Tn7 (RP4)(pAt396).

Table 6.2 Frequencies of transfer of RP4 and pAt396 between bacterial strains

Donor	Recipient	Marker selected	Transfer frequency per recipient	Co-transfer marker screened	No. of colonies tested	% co-transfer
E. coli J53(RP4)	A. tumefaciens 396::Tn7	RP4	N.D.*			
396::Tn7 (RP4) (pAt396)	C58rif^R	RP4	10^{-1}	[illegible]	100	1.7%
		Tn7	10^{-4}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-4}	pAt396	250	1.6%
	C58Clrif^R	RP4	10^{-1}	pAt396	850	2.6%
		Tn7	10^{-4}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-4}	pAt396	550	2.0%
Five 396::Tn7(RP4(pAt396) donors were each crossed with C58rif^R and C58Clrif^R						
C58rif^R::Tn7 (RP4) (pAt396) (no. 1)	C58nal^R	RP4	10^{-1}	N.D.	N.D.	N.D.
		Tn7	10^{-1}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-2}	pAt396	200	2.2%
C58rif^R::Tn7 (RP4) (pAt396) (no. 2)	C58nal^R	RP4	10^{-1}	N.D.	N.D.	N.D.
		Tn7	10^{-1}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-1}	pAt396	200	2.9%
C58rif^R::Tn7 (RP4) (pAt396) (no. 3)	C58nal^R	RP4	10^{-1}	N.D.	N.D.	N.D.
		Tn7	10^{-1}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-1}	pAt396	200	100%
C58Clrif^R::Tn7 (RP4) (pAt396) (no. 4)	C58nal^R	RP4	10^{-1}	N.D.	N.D.	N.D.
		Tn7	10^{-1}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-1}	pAt396	200	8%
C58Clrif^R::Tn7 (RP4) (pAt396) (no. 5)	C58nal^R	RP4	10^{-1}	N.D.	N.D.	N.D.
		Tn7	10^{-1}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-1}	pAt396	200	11%
C58nal^R::Tn7 (RP4) (pAt396) (no. 6)	C58ery^R	RP4	10^{-2}	N.D.	N.D.	N.D.
		Tn7	10^{-2}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-2}	pAt396	200	0%
C58nal^R::Tn7 (RP4) (pAt396) (no. 7)	C58ery^R	RP4	10^{-2}	N.D.	N.D.	N.D.
		Tn7	10^{-2}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-2}	pAt396	200	0%
C58nal^R::Tn7 (RP4) (pAt396) (no. 8)	C58ery^R	RP4	10^{-2}	N.D.	N.D.	N.D.
		Tn7	10^{-2}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-2}	pAt396	200	0.5%
C58nal^R::Tn7 (RP4) (pAt396)	C58ery^R	RP4	10^{-2}	N.D.	N.D.	N.D.
		Tn7	10^{-2}	N.D.	N.D.	N.D.
		RP4 and Tn7	10^{-2}	pAt396	200	11%
Donors no.'s 5, 7, 8 and 9 are [illegible]						

N.D. not done

* RP4 was transferred but the frequency was not determined.

Table 6.3 Transfer frequencies of RP4 from *Agrobacterium* to different *Rhizobium* species

Donor	Recipient	Marker selected	Transfer frequency per recipient	No. of colonies tested for agrocin production[1]	% co-transfer
C58rif^R ::Tn7 (RP4) (pAt396) (number 3)	RF6nal^R	RP4	1.4×10^{-4}	500	0.04
	RF10nal^R	RP4	1.0×10^{-4}	500	0.08
	RF14nal^R	RP4	2.0×10^{-6}	500	0
	RF22nal^R	RP4	2.0×10^{-5}	500	0
	VK10nal^R	RP4	1.0×10^{-5}	500	0
	WB1nal^R	RP4	1.0×10^{-5}	500	0
	TU1nal^R	RP4	1.75×10^{-6}	500	0.02
	SR4nal^R	RP4	2.7×10^{-5}	500	0
	SQ9nal^R	RP4	2.5×10^{-5}	500	0
	SACInal^R	RP4	5.0×10^{-5}	500	0

[1]Transconjugants carrying RP4 were screened for the co-transfer of pAt396 i.e. for the ability to produce agrocin.

tumefaciens, A. rhizogenes and A. rubi. This medium inhibits nearly all soil microflora, except Agrobacterium species against which it has no repressive effect. No rhizobia can grown on this medium. Only the two transconjugants having profiles resembling that of the donor were able to grow on this medium.

When mixtures of A. tumefaciens strain C58 and the agrocin producing Rhizobium transconjugants were inoculated on to potato slices, gall production was not inhibited. Even a five fold excess of the Rhizobium transconjugant over C58 cells did not prevent gall formation by C58.

6.5 DISCUSSION

The above results show that RP4 is capable of mobilising pAt396 to different *Agrobacterium* strains at significant frequencies. The mere presence, however, of RP4 in a strain does not necessarily result in mobilisation of pAt396. Mobilisation of pAt396 to *Agrobacterium* varied from < 0.2% to 11%. This shows the importance of testing a number of donor strains for mobilisation. The reason for the isolated case where 100% co-transfer of RP4 and pAt396 occurred is not known, but it is clearly not due to stable cointegrate formation. The possibility, however, of the formation of transient cointegrates, which resolve in the recipients cannot be completely ruled out.

Recent work by Hooykaas *et al.* (1981) has shown that Ti plasmids from *A. tumefaciens* introduced into *R. trifolii* and *R. leguminosarum* were expressed, but not in *R. meliloti*. Therefore different species were tested for their ability to inherit and express the plasmid carrying genes encoding agrocin production. Transfer of RP4 from *Agrobacterium* occurred at a lower rate than the transfer between *Agrobacterium* species. This can be expected since the frequency of transfer of RP4 between genera is lower. Although *R.*

meliloti and *R. leguminosarum* exconjugants were thought to be isolated, their profiles were not readily interpretable. Four of the *R. meliloti* transconjugants (transconjugant RF6nal^R number 1 and RF10nal^R numbers 1, 2 and 3) harboured the two plasmids of the recipients, RP4, as well as a plasmid with a lower molecular weight than pAt396. It is possible that this plasmid encodes agrocin production. One *R. meliloti* transconjugant (RF6nal^R number 2) produced agrocin but did not harbour pAt396 or a smaller plasmid similar in size to the one found in RF6 transconjugant number 1. Where the genes encoding agrocin production are located is not known. Two of the transconjugants, TU1nal^R and RF10nal^R transconjugant number 4, turned out to be *Agrobacterium* colonies.

The agrocin producing *Rhizobium* transconjugants were not able to control gall fomation on potato slices by the virulent strain C58. However, since cases have been reported where biological control was achieved by organisms *in vivo* but not *in vitro* (Moore and Warren, 1979), the *Rhizobium* transconjugants might not necessarily be useless in the field.

The study of the mobilisation of pAt396 by RP4 was quite extensive. It showed that RP4 can be transferred between *Agrobacterium* and *Rhizobium*. At

the same time mobilisation of pAt396 to Rhizobium occurred at extremely low frequencies, even though five different species of Rhizobium were tested, and five hundred RP4 carrying transconjugants from each cross were assayed for agrocin production. Rhizobium does not appear to be an acceptable host for the plasmid pAt396. It is likely that pAt396 is not readily transferred into the bacterium, or that pAt396 is unstable in Rhizobium.

This work also shows that, using the method of Kado and Liu(1981), to extract plasmids, it is important to heat treat different bacteria at 95°C for different lengths of time since under and overtreatment with heat results in degradation of large plasmids.

Thus, although an agrocin producing Rhizobium strain which is able to control crown gall was not produced, this work may still be of academic interest.

SUMMARY AND DISCUSSION

Various means of biological control of crown gall, a neoplastic disease of dicotyledonous plants caused by *A. tumefaciens* are being sought. Strain K84 has been used successfully in several countries(Kerr and Htay, 1974). However, many local isolates of *A. tumefaciens* are resistant to agrocin 84 and strain 84, being an *Agrobacterium* strain, can be a potential pathogen.

Two approaches were taken in trying to overcome part of this problem. The first approach taken was to screen local isolates of *A. tumefaciens*, isolated around gall areas on plants, for new agrocins especially those which have a broad host range. The basis for this was that agrocins are very specific as to the host against which they are active, for example, agrocin 84 is only able to act against *Agrobacterium* strains belonging to biotype 1 and 2 harbouring a nopaline Ti plamid. Different types of Ti plasmids exist (based on their ability to metabolise a specific opine), and hence it has been proposed(Kerr and Htay 1974) that different agrocins should exist which can act on the different Ti plasmid types. The second approach was to transfer the agrocin encoding plasmid, pAt396, from *A.*

tumefaciens to Rhizobium species. The reason for choosing Rhizobium was that it is non-pathogenic, closely related to Agrobacterium, and is an inhabitant of the rhizosphere, and hence shares the same environment as Agrobacterium.

Only one local strain, D286, belonging to biotype 1, exhibited a broader host range than agrocin 84. Agrocin D286 inhibited the growth of A. tumefaciens strains harbouring a nopaline, octopine or agropine type Ti plasmid, whereas agrocin 84 is only active against strains harbouring a nopaline Ti plasmid(Kerr and Roberts, 1976). Laboratory tests carried out on potato slices on the ability of D286 to inhibit crown gall formation by Ti plasmid carrying strains were successful. Agrocin D286 inhibited the formation of galls by C58, Ach5 and D208. This occurred even when there was an eight-fold excess of C58 over D286 cells and a five fold excess of D208 cells over D286. However, a 1:1 ratio was required for control of gall formation by Ach5. Similarly, a 1:1 ratio of strain 84 to the pathogen, C58 was necessary for biological control. When equal numbers of heat treated D286 cells were mixed with the pathogen, gall formation was not inhibited, indicating that agrocin production by viable cells, and not cell competition for infection sites was necessary for the inhibition of gall formation.

The genetic characteristics of production of agrocin D286 were studied. Much higher levels of agrocin were produced in liquid culture by D286 than by 396::Tn7 (Figure 3.1). However, the activity of agrocin D286 rapidly disappeared on further incubation of the culture. The loss of agrocin activity was not due to instability since agrocin in the supernatant fluid (crude agrocin) was stable after pelleting of the cells. No agrocin degrading activity was associated with cells. It has been suggested that a cometabolite is produced concomitantly with the agrocin which is responsible for agrocin degradation but which is unable to act on the agrocin after subsequent addition to it. The loss of agrocin activity does not appear to be a problem in controlling gall formation since strain D286 effectively inhibited tumor formation by virulent strains on potato slices. The time course of production of agrocin by D286 followed similar patterns in LB and minimal broth containing glucose or sodium glutamate. However much larger zones of inhibition against a sensitive strain were produced on MM plates containing sodium glutamate compared to MM containing glucose. This may be attributed to the effect of glucose on the sensitivity of the test strain to the agrocin(Moore and Warren, 1979), or to the fact that a larger amount of agrocin was produced

on solid medium in the presence of sodium glutamate. Different degrees of sensitivity were found to be exhibited by different agrocin sensitive strains, for example, a larger zone of inhibition was constantly observed around strain D286 when strain D208 was use as the sensitive strain (Figure 3.3) than when C58 (Figure 3.4) was used as the sensitive strain even though both plates were exposed to the same conditions. Unlike other bacteriocins produced by *Serratia* and *Listeria* for example(Mayr-Harting *et al*., 1972), agrocin D286 production was not induced by mitomycin C or ultraviolet light.

Strain D286 was originally isolated as a pathogenic strain. However, when it was found to produce an agrocin and reinoculated on to indicator plants and potato slices, this strain appeared to be non-pathogenic. The discovery that strain D286 had lost its pathogenicity was surprising. Plasmid analysis revealed the presence in strain D286 of a cryptic plasmid (approximately 300 Md) as well as a smaller plasmid with a lower molecular weight than the Ti plasmid but with a higher molecular weight than pAt84a (Figure 3.9). This plasmid might be a Ti plasmid with regions encoding oncogenicity deleted, or it could encode agrocin D286 production. Strain D286 was not cured of its ability to produce agrocin using curing agents mitomycin C, acridine orange,

acriflavine, coumermycin or ethidium bromide. However, if the plasmid does encode agrocin production then it is not surprising that D286 was not cured of this function as large plasmids are extremely difficult to cure. Strain 396::Tn7 was not cured of pAt396 which is considerably smaller than the plasmids in D286 (Figure 3.9), and neither was C58 cured of pTiC58.

The physical characterisitics of partially purified D286 agrocin and particularly its UV absorption spectrum (Figure 4.1), are consistent with its being a nucleotide, as is agrocin 84. Agrocin 84 is a disubstituted fraudulent nucleotide with a 9-(3'-deoxy-α-D-threo pentafuranosyl) adenine core. The crude and partially purified agrocin was not inactivated by enzymes DNase I, RNase A or protease. Both preparations were stable at pH ranging from 3 to 9, and at 26°C but not at 37°C. The modes of action of agrocin D286 and 84 appear to be similar. D286 agrocin was found to inhibit DNA, RNA and protein synthesis of sensitive D208 cells. Das et al.(1978) showed that agrocin 84 inhibited DNA synthesis of sensitive cells at low concentrations, and RNA and protein synthesis only at higher concentrations. However, there is still controversy as to the mode of action of agrocin 84.

Sensitivity to agrocin D286 was found to map in the 11 to 18 Md region of the nopaline Ti plasmid. Use was made of pTiC58 plasmids from which known regions are deleted. Exclusion of bacteriophage AP1 was also found to map in this region. Whether there is any relationship between the two functions has not been determined. Agrocin D286 was also active against strains harbouring an octopine Ti plasmid. Sensitivity to agrocin D286 was found to coincide with the B region of homology between the nopaline and octopine Ti plasmids (Figure 1.3).

The agropine Ti plasmid of strain D208 was found to be responsible for agrocin D286 sensitivity. Virulence tests and plasmid screening revealed that strain D208 had been cured of its Ti plasmid (Figure 5.1) at 37^{0}C. This strain was resistant to agrocin D286.

The analysis of sensitivity to agrocin D286 of strains harbouring mutant Ti plasmids or of strains cured of the Ti plasmid was initially hampered by the occasional appearance of faint zones of inhibition of some strains when tested by the method of Kerr and Htay(1974). This was particularly noticeable after substituting glucose in the medium with sodium glutamate. From the results (Table 5.2), it appears that while sensitivity to agrocin D286 is indeed

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encoded by the Ti plasmid, another plasmid-independent uptake system may exist which is considerably less efficient and only rarely noticeable.

A number of conjugation steps were performed in order to determine whether RP4 could mobilise pAt396 either as a pAt396::RP4 cointegrate or as two separate entities. RP4 was initially introduced into an agrocin producing strain of A. tumefaciens 396::Tn7(pAt396) from E. coli J53(RP4). Profiles of the plasmids in the donor, J53(RP4), recipient, 396::Tn7 and the transconjugant, 396::Tn7(RP4)(pAt396) (Figures 3.2a and b, 3.3a and b, and 3.4a and b) proved that the transfer of RP4 had occurred. RP4 was subsequently transferred from 396::Tn7(RP4)(pAt396) to C58rif^{R} and C58Clrif^{R}, and then to C58nalR and C58eryR. In each of the cases the percentage coinheritance of pAt396 was determined Plasmid profiles of donor, recipient and transconjugant bacteria revealed the transfer of pAt396 and RP4 in each case.

Plasmid RP4 was transferred between A. tumefaciens strains at a high efficiency, in the range of 10^{-1} and 10^{-2} per donor and per recipient. A cointegrate RP4::pAt396 plasmid was not formed in Agrobacterium. However, RP4 successfully mobilised pAt396 between

Agrobacterium strains although this occurred at varying frequencies from 0.5% to 100%. In some of the cases pAt396 was not mobilised at all. The presence of the Ti plasmid did not affect transfer frequency of RP4 or pAt396 since both C58 and C58C1 received RP4 and pAt396 at similar frequencies. Since no cointegrate RP4::pAt396 plasmid had been detected, C58rif^R::Tn7(RP4)(pAt396) number 3 (Table 6.2) which carried RP4 with a high mobilising ability (i.e. which mobilised pAt396 to C58nal^R with 100% efficiency) was used in matings with Rhizobium strains.

Plasmid RP4 was transferred to Rhizobium species at a rate of between 10^{-4} and 10^{-6} per recipient. Out of five thousand Rhizobium transconjugants tested, only seven were found to produce agrocin. Four of these transconjugants resulted from a cross using R. meliloti RF10nal^R as recipient, two using RF6nal^R, and one using TU1nal^R. The plasmid profiles of the transconjugants were, however, difficult to interpret. Three of the RF10nal^R transconjugants (numbers 1, 2 and 3; Figure 13 and 14) and one RF6nal^R transconjugant (number 1; Figure 6.11) harboured four plasmids; two of them were the same as those found in the recipients, RP4, and a plasmid of lower molecular weight than pAt396. It is not known whether this plasmid encodes agrocin. One RF6 transconjugant

(number 2; Figure 6.12) only harboured the two recipient plasmids and RP4. The location of the genes encoding agrocin production in this strain is not known. One RF10 transconjugant (number 4; Figure 6.14) and the TU1 transconjugant (Figure 6.15) were found to be Agrobacterium isolates which had spontaneously become rif^S and nal^R. The two isolates harboured the same plasmids as those found in the donor C58rif^R::Tn7(RP4)(pat396). They were able to produce galls on potato slices, and to grow on media which specifically selects for Agrobacterium.

Many steps were taken to obtain a Rhizobium strain which could produce agrocin and be effective in controlling crown gall formation. Different Rhizobium species were used in the conjugation experiments, as it has previously been shown (Hooykaas et al., 1981) that certain Rhizobium species viz. R. trifolii and R. leguminosarum carrying the Ti plasmid were able to express Ti plasmid genes, whereas R. meliloti was unable to express these genes. Also a statistically suitable number of RP4 carrying Rhizobium transconjugants (five hundred colonies from each of ten crosses) were tested for their ability to produce agrocin. However, although our aim was not completely achieved, these results may well be of interest academically.

In conclusion, I have isolated a new non-pathogenic agrocin producing strain of A. tumefaciens with a broad host range, which may be useful in biological control of crown gall. Laboratory tests on potato slices show that strain D286 inhibits gall formation by C58, Ach5 and D208 strains which are sensitive to its agrocin. Strain D286 could possibly be used together with strain K84 in biological control. Most Rhizobium strains are resistant to agrocin D286. Only two out of sixteen were found to ' sensitive. These ·o strains are not used commercially. Therefore it appears as though agrocin D286 will not pose any problem as far as the nitrogen fixing bacteria are concerned. Strain D286 is also useful since it is non-pathogenic. The advantages of strain D286 over strain K84 are its activity against strains harbouring nopaline, octopine, and agropine type Ti plasmids, as well as its faster growth rate and its ability to produce high levels of agrocin. As poor growth rate of an agrocin producing strain in vivo might be a reason for lack of biological control (Moore and Warren 1979) a strain with a fast growth rate which produces a high level of agrocin might well be advantagous. A disadvantage with strain D286 is the fact that agrocin activity is rapidly lost if not removed from the producing cells. However this might merely be a laboratory observation which does not occur in planta. It remains to be

determined whether strains D286 and K84 can complement each other in the control of crown gall.

APPENDIX I

MEDIA AND BUFFERS

Agrobacterium minimal medium A

Salts solution:	K_2HPO_4	10.5 g
	KH_2PO_4	4.5 g
	Tri-sodium citrate	0.5 g
	$(NH_4)_2SO_4$	1.0 g

Above salts are dissolved in 200 ml distilled water and sterilised by autoclaving.

$MgSO_4$: 10% (w/v) is sterilised by autoclaving.

Vitamin B1: 1% (w/v) is sterilised by filtration.

Glucose: 20% (w/v) is sterilised by autoclaving.

Sodium glutamate: 20% (w/v) is sterilised by autoclaving.

Water agar:	Oxoid agar No. 3	10 g
	Distilled water	800 ml

Sterilise by autoclaving

To make up:	Water agar	800 ml
	Salts solution	200 ml
	$MgSO_4$	0.1 ml
	Vitamin B1	0.1 ml
	Glucose	1 ml

Glucose can be substituted by 10 ml sodium glutamate.

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Glucose:	20% (w/v) is sterilised by autoclaving.
Sodium glutamate:	20% (w/v) is sterilised by autoclaving.

Water agar:	Oxoid agar No. 3	10 g
	Distilled water	800 ml

Sterilise by autoclaving

To make up:	Water agar	800 ml
	Salts solution	200 ml
	$MgSO_4$	0.1 ml
	Vitamin B1	0.1 ml
	Glucose	1 ml

Glucose can be substituted by 10 ml sodium glutamate.

E Buffer

Trizma base	40 mM
Sodium EDTA	2 mM

Adjust to pH 7.9 with acetic acid

Luria agar

Luria broth	1 l
Oxoid agar No. 3	10 g

Luria broth

Oxoid tryptone	10 g
Oxoid yeast extract	5 g
NaCl	10 g
Distilled water	1 l

Saline

NaCl	8 g
Distilled water	1 l

Scintillation cocktail

Toluene	12.5 g
PPO	2.5 l

Tracking dye

Bromophenol blue	0.025 g
Glycerol	5 ml
0.1M Tris-acetate	5 ml

pH 7.9

TY agar (Beringer, 1974)

TY broth	1 l.
Oxoid agar No. 3	10 g

TY broth

Difco tryptone	5 g
Difco yeast extract	3 g
$CaCl_2$	7 mM
Distilled H_2O	1 l

YMA

Mannitol/Maltose	10 g
Difco yeast extract	1 g
$K_2HPO_4.3H_2O$	0.65 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Oxoid agar No. 3	12 g
Distilled H_2O	1 l

Agrobacterium growth medium

Glycerol	1 ml.
KNO_3	0.1 g
$MgSO_4.7H_2O$	0.01 g
Na_2HPO_4anh.	0.018 g
$CaCl_2$	0.004 g
Oxoid agar No. 3	1.2 g
Distilled H_2O	100 ml

The above solution is sterilised by autoclaving, and the following added aseptically.

Fe EDTA	(0.25% w/v)	1 ml
Biotin		25 µg
Thiamine		100 µg

The pH was adjusted to 6.8.

Agrobacterium selective medium

Lactose	0.5 g
KNO_3	0.1 g
$MgSO_4.7H_2O$	0.01 g
Na_2HPO_4 anh.	0.018 g
Oxoid agar No.3	1.2 g
Distilled H_2O	100 ml

The above solution is sterilised by autoclaving, and the following added aseptically.

Fe EDTA	(0.25% w/v)	1 ml
$MnSO_4.\ 4H_2O$	(33.5%)w/v)	1 ml

The pH was adjusted to 6.8.

APPENDIX II

SOURCES OF CHEMICAL REAGENTS

Acridine orange	Sigma
Acriflavine	Sigma
[^{3}H] adenine	Amersham
Agar technical no. 3	Oxoid
Agarose	Seakem
Ammonium phosphate	Riedel-De Haen Ag Seelze-Hannover
Ammonium sulphate	Saarchem
Biotin	Sigma
Bromophenol blue	Merck
Calcium chloride	Merck
Coumermycin	Bristol Laboratories
Charcoal	Riedel-De Haen Ag Seelze-Hannover
DEAE Sephadex	Pharmacia
Deoxyribonuclease I	Sigma
2,5-Diphenyloxazole	Sigma
Dipotassium hydrogen orthophosphate	Merck
Disodium hydrogen orthophosphate	Merck
Erythromycin	Sigma
Ethidium bromide	Sigma
Ethylenediaminetetra-acetic acid	BDH
Ferric ethylenediamine tetra-acetic acid	Sigma

Ficoll	Sigma
Glucose	BDH
Glycerol	Merck
Kanamycin sulphate	Sigma
[^{14}C] leucine	Amersham
Magnesium sulphate	Saarchem
Manganese sulphate	Analar
Mitomycin C	Sigma
Naladixic acid	Sigma
Potassium dihydrogen orthophosphate	Merck
Potassium nitrate	SMM
Phenol	SMM
Protease	Sigma
Ribonuclease A	Sigma
Rifampicin	Sigma
Sodium chloride	Saarchem
Sodium glutamate	BDH
Sodium hydroxide	SMM
Sodium dodecyl sulphate	Merck
Spectinomycin sulphate	Sigma
Streptomycin sulphate	Novo industries
Thiamine	BDH
[^{3}H] thymine	Amersham
Trichloroacetic acid	Merck
Tri-sodium citrate	Merck
Trizma base	Sigma
Tryptone	Oxoid
Vitamin B1	BDH
Yeast Extract	Difco/Oxoid

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