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GENETIC ASPECTS OF THE INDUCTION AND BIOLOGICAL CONTROL OF CROWN GALL

by

JEFFREY GRAHAM ELLIS
B.Ag.Sc. (Hons.) Adelaide

Department of Plant Pathology
Waite Agricultural Research Institute
University of Adelaide
South Australia

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SUMMARY

Genetic aspects of arginine catabolism in *Agrobacterium radiobacter* were studied. At least one step in the degradation of arginine to supply a carbon source is controlled by the octopine and nopaline Ti plasmids. Ti plasmid linked arginine catabolism is expressed when strains harbouring octopine or nopaline Ti plasmids are induced by the respective opine but not by arginine itself. This character is also expressed by strains harbouring Ti plasmids with regulatory mutations of the octopine or nopaline catabolic system. The ability to catabolise arginine was transferred with the Ti plasmids to recipient strains and provided a useful selective marker for the plasmids when the recipient was unable to catabolise arginine.

A second form of transmissible arginine catabolism was studied in octopine strains NCPPB 1001 and R10. Although transfer was mediated by the octopine Ti plasmid, it frequently occurred without transfer of this plasmid to the transconjugant. An unsuccessful search was made for a hypothetical plasmid controlling arginine catabolism that was mobilised from the donor by the Ti plasmid. Since the techniques used could detect plasmids of molecular weights up to 300×10^6 daltons, the possibility was considered that the Ti plasmid can mobilise chromosomally-linked arginine catabolic genes.

Further evidence is presented that biological control of crown gall by *A. radiobacter* var. *radiobacter* strain 84 is due to production of a bacteriocin, agrocin 84. The biosynthesis of agrocin 84 is

controlled by a $30x10^6$ dalton plasmid, pAt-84a. When this plasmid was transferred from the donor strain 84 to avirulent recipients, most transconjugants inherited the ability to produce agrocin 84 and the ability to act as biological control agents. This plasmid was also transferred to pathogenic strains. These strains produced agrocin 84 and were immune to its action. Such genetic transfers occurring in the field would threaten the effectiveness of this method of biological control. The bacteriocinogenic plasmid also confers immunity on its host to agrocin 84 and to a toxic degradation product of agrocin 84 described by Tate *et al.* (1979). This product lacks the bacteriocin-like specificity of agrocin 84. Immunity to it is a selectable trait of pAt-84a that is expressed even in the absence of the Ti plasmid-linked agrocin sensitivity marker. Further genetic studies of pAt-84a will therefore be facilitated.

The genetic basis of agrocin production in several other strains was investigated. Agrocin production by strains NCPPB 398 and Bo542 is also controlled by plasmids of similar size to pAt-84a.

Agrocin 84 sensitivity (Agr^S) in *Agrobacterium radiobacter* is due to a nopaline Ti plasmid-linked permease (Murphy and Roberts, 1979). A search for a non-toxic substrate for this permease revealed a new group of crown gall specific metabolites called agrocinopines. Two members of this group, agrocinopines A and B, occur in tumours induced by nopaline strains and two others, agrocinopines C and D, in tumours induced by agropine strains. Synthesis and catabolism of these compounds is controlled by Ti plasmid-linked genes.

The agrocinopines are phosphorylated sugar derivatives. Sucrose, phosphorous, arabinose and glucose were identified in hydrolysates of

agrocinopine A and sucrose, phosphorous and glucose were identified in hydrolysates of agrocinopine C. Agrocinopine A can be readily converted to agrocinopine B by loss of glucose and agrocinopine C was converted to agrocinopine D presumably by loss of fructose. Therefore agrocinopines B and D may be artifacts of purification of agrocinopines A and C respectively.

Agrocinopines A and B induce the conjugative transfer of nopaline Ti plasmids and agrocinopines C and D induce transfer of agropine Ti plasmids. Mutant plasmids constitutive for transfer (Tra^c) can be isolated in transconjugants when the donor has not been induced by agrocinopines. A study of several Tra^c mutants of pTi-C58 revealed several interesting properties. Tra^c strains are constitutive for uptake of agrocinopine A. Tra^c mutant plasmids also confer on their hosts supersenstivity to agrocin 84 due to an increased uptake of the bacteriocin. When such a Tra^c plasmid is harboured in a cell that also contains the bacteriocinogenic plasmid pAt-84a, agrocin 84 biosynthesis does not occur. The ability to "turn-off" agrocin 84 biosynthesis is controlled by genes at or near the Agr^s (agrocin 84 sensitivity) region of the Ti plasmid. When a Tra^c plasmid carries an Agr^r mutation, agrocin 84 biosynthesis occurs. Possible mechanisms for this process are discussed.

A study of 79 Agr^r mutants of a Tra^c nopaline Ti plasmid was carried out. Some of these plasmids carried small deletions that could be detected by agarose gel electrophoresis. Three phenotypic classes were conferred by these Agr^r mutant plasmids, namely $Agr^rTra^+Onc^+$, $Agr^rTra^-Onc^-$ and less frequently $Agr^rTra^-Onc^+$. These data confirm

the results of Holsters *et al.* (1980) who showed that Onc and Tra functions map in the Agr^S region of the nopaline Ti plasmid, pTi-C58.

STATEMENT

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

J.G. ELLIS

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CHAPTER 1

GENERAL INTRODUCTION

Crown gall is a disease that affects many dicotyledonous plants and is manifested by tumorous growths on the roots, crowns and sometimes on aerial parts of plants. The pathogenic organism that causes crown gall is a soilborne bacterium (Smith and Townsend, 1907) of the genus *Agrobacterium*. This species has been designated *A. radiobacter* with varietal epithets *radiobacter* or *tumefaciens*, depending on whether the strain is non-pathogenic or pathogenic (Keane, Kerr and New, 1970). Three subgroups or biovars have been recognised; biovars 1 and 2 (Keane, Kerr and New, 1970) and biovar 3 (Kerr and Panagopoulos, 1977).

Crown gall has the distinction of being the best understood plant disease in terms of its molecular biology and biological control. Many researchers became interested in crown gall because it is a plant cancer. White and Braun (1942) showed that crown gall cells could be maintained in tissue culture without a cupply of exogenous phytohormones and in the absence of the pathogenic strain of *A. radiobacter*.

Braun (1947) proposed that the bacteria produced a 'tumour-inducing principle' that causes the tumorous transformation of the plant cell. Much research has been carried out to determine the nature of this substance.

Prior to 1969, the most successful approach to crown gall research relied on the development of methods for culturing plant tissue

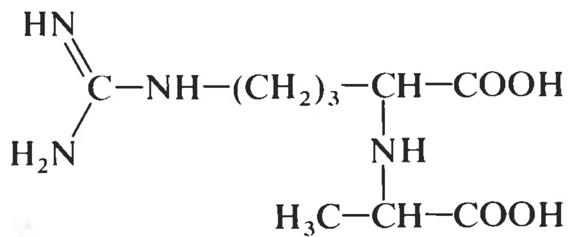
in vitro and on the search for differences between normal and crown gall tissue cultures.

Morel's group in Versailles (Ménagé and Morel, 1964; Goldman *et al.*, 1969) used this approach and discovered two arginine drivatives, octopine and nopaline (see Figure 1-1) in crown gall tissue. These substances are not found in normal plant tissue (Scott *et al.*, 1980). A lysine derivative, lysopine which was also crown gall tissue specific had been discovered earlier (Lioret, 1956) and several other similar crown gall specific compounds (Figure 1-1) were discovered later, but the work on octopine and nopaline was the most significant in the development of our understanding of crown gall.

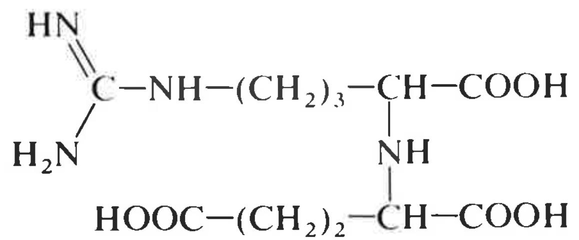
Goldman *et al.* (1968) showed that whether nopaline or octopine was produced in sterile crown gall tissue culture depended on the strain of bacterium used to induce the tumour. Petit *et al.* (1970) extended this observation to a larger sample of strains of *A. radiobacter* and demonstrated that those strains that induced tumours containing nopaline could catabolise nopaline and those that induced octopine galls could catabolise octopine. Two groups of strains, nopaline strains and octopine strains, could therefore be recognised. Petit *et al.* (1970) proposed that the tumorous transformation of plant cells may result from the transfer of genetic information from the bacterium to the plant. Their insight had resulted from a close study of both crown gall plant tissue and of the pathogenic organisms.

Kerr (1969) reported experiments in which virulence was transferred from a genetically marked pathogenic donor to a non-pathogenic

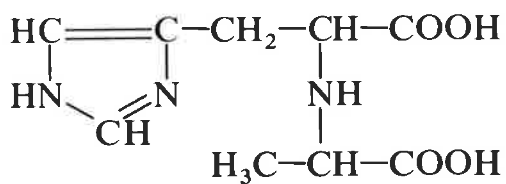
Figure 1-1. The pyruvic acid derived opines, octopine, octopinic acid, lysopine and histopine are synthesised by tumours induced by strains of *Agrobacterium radiobacter* harbouring octopine Ti plasmids. The α-ketoglutaric acid derived opines, nopaline and nopalinic acid are synthesised by tumours induced by nopaline strains.



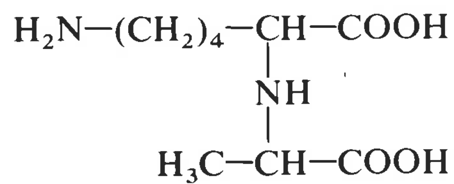
octopine



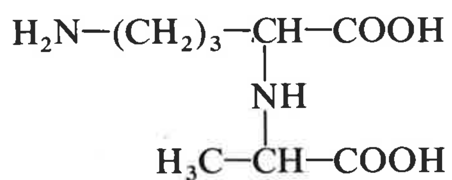
nopaline



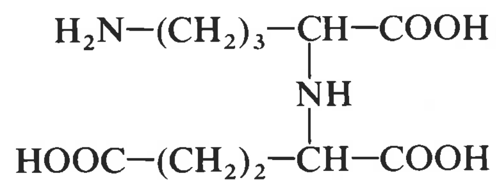
histopine



lysopine



octopinic acid



nopalinic acid

recipient. Further data on virulence transfer which occurred in mixed infections of donor and recipient in crown gall tissue were reported in 1971 (Kerr, 1971). Virulence was obviously associated with a transmissible element. In 1974, Zaenen *et al.* demonstrated a strong correlation between the presence in *A. radiobacter* of a large plasmid and the ability to induce crown gall. In the same year, Van Larebeke *et al.* (1974) showed that the loss of virulence by strain C58 during culture at 37°C, which was first reported by Hamilton and Fall (1971), was associated with the loss of a large plasmid. Using the methods of Kerr (1969, 1971), Van Larebeke *et al.* (1975) and Watson *et al.* (1975) demonstrated that virulence transfer was associated with the transfer of a large plasmid. These plasmids are now called Ti plasmids.

The Ti plasmids are large, with molecular weights in the vicinity of 120×10^6 daltons, which gives them the coding capacity of approximately 200 average sized proteins (Kerr, 1978). In addition to virulence, several other properties have been ascribed to the Ti plasmids. One group of Ti plasmids carries the genes which determine the synthesis of octopine and associated pyruvic acid derivatives in the tumour and also those controlling octopine catabolism by *A. radiobacter*; the second group determines nopaline and nopalinic acid synthesis and breakdown (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975; Bomhoff *et al.*, 1976; Montoya *et al.*, 1977). These two groups are referred to as octopine and nopaline Ti plasmids. The nopaline Ti plasmids also carry the gene(s) that encode sensitivity to agrocin 84 (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975; Engler *et al.*, 1975),

which is a nucleotide bacteriocin produced by strain 84 of *A. radiobacter* var. *radiobacter* (Tate *et al.*, 1979). A third but minor group of Ti plasmids was recognised by Montoya *et al.* (1977); strains harbouring these plasmids can catabolise neither octopine nor nopaline and induce tumours containing neither of these compounds. This group was referred to as 'null' type Ti plasmids, but are now called agropine Ti plasmids, since they carry genes that induce synthesis and catabolism of agropine (Guyon *et al.*, 1980), which is a crown gall specific amino acid-sugar derivative (Firmin and Fenwick, 1979; Coxon *et al.*, 1980). These three types of plasmid can also be grouped on the basis of their DNA homology (Currier and Nester, 1976) and their SmaI restriction endonuclease digest patterns (Sciaky *et al.*, 1977).

The Ti plasmids are also conjugative plasmids (Kerr *et al.*, 1977; Genetello *et al.*, 1977). Transfer functions of octopine plasmids are normally repressed but can be induced by octopine (Petit *et al.*, 1978a; Klapwijk *et al.*, 1978). The genetics of transfer of nopaline and agropine Ti plasmids is a subject of this thesis (Chapter 7). The discovery of transfer of Ti plasmids on agar media opened the way for more sophisticated genetic approaches to the study of these plasmids and the genetics of crown gall. Mutagenesis of Ti plasmids by drug resistance transposon insertion (Hernalsteens *et al.*, 1978) and physical mapping of Ti plasmids by restriction endonuclease digest analysis (Chilton *et al.*, 1977; Depicker *et al.*, 1980) has enabled the development of physical and genetic maps of Ti plasmids (Holsters *et al.*, 1980).

Since Petit *et al.* (1970) proposed that crown gall tumour induction may involve the transfer of genetic information from the bacterial pathogen to the plant, many attempts have been made to show that there was bacterial DNA in sterile crown gall tissue. Chilton *et al.* (1977) were the first to show that bacterial DNA or, more specifically a small segment of the Ti plasmid, could be detected in crown gall tissue but not in normal plant tissue. This DNA segment, called T-DNA, was detected in DNA extracted from sterile tumour tissue by studying the kinetics of reassociation of single stranded total tumour DNA and ^{32}P-labelled probe DNA comprising SmaI digest fragments of the Ti plasmid isolated from the bacterium that had induced the tumour. This work has been confirmed by the Belgium Crown-gall Research Group using ^{32}P-labelled restriction fragments of Ti plasmids as probes and the 'Southern blotting' hybridization technique to demonstrate the presence of DNA homologous to Ti plasmid sequences in crown gall tumour tissue (Schell *et al.*, 1979.

T-DNA encompasses a sequence of DNA that is common to all Ti plasmids that have been analysed (Chilton *et al.*, 1978; Depicker *et al.*, 1978). This common DNA is essential for virulence. Insertion of the drug resistance plasmid RP4 or transposons into this region results in a loss of virulence (Depicker *et al.*, 1978; Holsters *et al.*, 1980). The evolutionary conservation of this region indicates that there may be a single mechanism determined by these Ti plasmids for induction and maintenance of the tumorous state.

Further evidence that T-DNA is transferred from bacterial to plant cell during tumour initiation has been reported by Schell *et al.*

(1979) and Holsters *et al.* (1980). The drug resistance transposon was inserted into various locations in the Ti plasmid, pTi-T37 and the strains harbouring the transposon were used to induce tumours. One mutant induced tumours that did not synthesise nopaline. The transposon Tn7 was mapped in the T-DNA of the Ti plasmid of this strain and located by Southern blotting in the DNA of sterile tumour tissue induced by this mutant. Apart from showing that DNA transfer had taken place, this result defines the location of the gene that directs nopaline biosynthesis.

Regions of the T-DNA are transcribed in crown gall tissue. Drummond *et al.* (1977) isolated ^{32}P pulse labelled RNA from tumour and normal callus tobacco tissue cultures and hybridized it to restriction digest fragments of the Ti plasmid that had incited the tumour. Their Southern hybridizations showed that RNA from the tumour tissue, but not the normal tissue, was homologous to part of the Ti plasmid maintained in crown gall tissue. The data showed that the T-DNA was transcribed. Similar conclusions were reached by Ledeboer (1978), Gurley *et al.* (1979), Yang *et al.* (1980), and by Willmitzer *et al.* (1980). All these experiments confirm the presence of T-DNA in tumour cells using an approach independent of that used by Chilton *et al.* (1977).

The means by which the T-DNA is transferred to the plant cell and its location within the plant genome is still unknown. Chilton *et al.* (1980) and Willmitzer *et al.* (1980) analysed the DNA of mitochondria and chloroplasts of crown gall tissue for T-DNA with negative results. They concluded that T-DNA is not located in plastid DNA and may therefore be found in nuclear DNA or in as yet unidentified

extrachromosomal DNA molecules. Nevertheless, they and other workers (Schell *et al.*, 1979; Thomashow *et al.*, 1980a) have shown that when tumour cell DNA is extracted and cut with restriction enzymes, some of the resulting fragments containing T-DNA are not completely homologous to probes made from Ti plasmid DNA. These observations can be accounted for if the T-DNA is covalently linked to plant DNA. So it appears that T-DNA is integrated into the plant genome. This has recently been confirmed by Yadav *et al.* (1980) and Zambryski (1980) who isolated and cloned 'border fragments' from crown gall teratoma tissue comprising T-DNA which is covalently linked to plant DNA.

The unusual plant metabolites, such as octopine and nopaline, found in crown gall tumours do not have a role in tumour induction or maintenance of the tumorous strate. This has been demonstrated by isolating mutant strains of bacteria which no longer induces the synthesis of these substances in the tumours (Klapwijk *et al.*, 1978; Koekman *et al.*, 1979; Holsters *et al.*, 1980). Rather, these compounds referred to as opines, have a nutritional role for the pathogen. Opines have been defined as compounds whose biosynthesis in crown gall tissue is directed by T-DNA and that can be catabolised via Ti plasmid encoded pathways to supply a source of nutrients to the Ti plasmid-harbouring bacterium (Petit *et al.*, 1978b; Schell *et al.*, 1979). Some opines also induce Ti plasmid transfer functions (Petit *et al.*, 1978a, and Chapter 6). Octopine and nopaline are important examples of opines. They are both arginine-keto acid condensation products and can be used as sole source of nitrogen and carbon by bacteria harbouring octopine and nopaline Ti plasmids respectively. Octopine also induces conjugal transfer of octopine Ti plasmids. So, in the environment of the crown gall tumour, opines

promote growth of bacteria harbouring a Ti plasmid and some induce the spread of the Ti plasmid throughout a bacterial population.

Virulent strains of *A. radiobacter* therefore create a favourable environment for themselves by transferring to the plant, genetic information which encodes the synthesis of opines in the tumour tissue. In this way, the photosynthetic potential of the plant is tapped and nutrients are supplied to *A. radiobacter* in a form that is available to few, if any, other organisms. This form of host-parasite relationship has been referred to as 'genetic colonisation' (Schell *et al.*, 1979). The opine concept is an important theme of this thesis.

In the field, crown gall is an important disease of many crops, particularly those in the Rosaceae. The most common pathogens isolated in this situation are *A. radiobacter* strains of biovar 2 harbouring nopaline Ti plasmids. This form of crown gall is now effectively controlled biologically by dipping planting material into a living cell suspension of the avirulent strain 84 of *A. radiobacter* var. *radiobacter*. This strain produces agrocin 84 and it is this toxic molecule that is believed to be the active component in biological control (Kerr and Htay, 1974). The genetics of agrocin 84 biosynthesis and biological control of crown gall and the relationship between biological control and the opine concept is a second major theme of this thesis.

CHAPTER 2

MATERIALS AND METHODS

In this chapter, materials and methods are described that have been used generally throughout this work. Specific experimental details that are relevant only to certain chapters are described in those chapters.

A. BACTERIAL STRAINS

A description of the bacterial strains that were used is presented in Table 2-1. The taxonomy of the genus *Agrobacterium* is confused in the literature. Virulence has been used as a criterion for differentiating two species within the genus, namely the virulent species *A. tumefaciens* and the avirulent species *A. radiobacter* (Allen and Holding, 1974). It is now considered inappropriate that a character encoded by a conjugative plasmid should be used to differentiate species of bacteria (Kerr *et al.*, 1978). Keane *et al.* (1970) proposed that there should be one species, *A. radiobacter*, with varietal epithets, *radiobacter* and *tumefaciens*, the latter epithet for virulent and the former for avirulent strains. The species can be further subdivided into 3 biovars by biochemical tests (Keane *et al.*, 1970; Kerr and Panagopoulos, 1977). For convenience, in this thesis all strains are referred to as *A. radiobacter* and the virulence of the strain and its biovar classification is indicated in Table 2-1 or in the text.

Table 2-1

Bacterial Strains

Strain	Biovar	Plasmid Type	Description and Origin
K11(MEL3)	1	Ti Nop*	R. Mushin
K14	1	"	Almond tree, S. Aust.
K19	1	"	Peach gall, S. Aust.
K31	1	"	Prunus gall, S. Aust.
K120	1	"	Cherry gall, Victoria
K122	1	"	Peach gall, Victoria
IIBV7	1	"	G. Morel, Versailles
C58	1	"	J. Schell, Ghent
K230	1	"	C58, R. Hamilton, Pennsylvania
H100	1	"	J. Tempé, Versailles
T37	1	"	G. Morel, Versailles
K27	2	"	Peach gall, S. Aust.
K105	2	"	Almond gall, S. Aust.
K108	2	"	Almond gall, S. Aust.
Ag43	2	"	C. Panagopoulos, Greece
K84	2	NOC†	S. Aust.
K112	2	NOC	S. Aust.
Bo542	1	TiAg**	Sciaky *et al.* 1977
NCPPB396	1	"	NCPPB
NCPPB398	1	"	NCPPB, Kerr and Roberts 1977
C58C1	1	cryptic	J. Schell, C58 cured of Ti plasmid
K57	1	cryptic	Potting soil, S. Aust.
K305	3	Ti Oct***	Vine gall, S. Aust.
K308	3	"	Vine gall, S. Aust.
NCPPB1001	1	Oct	NCPPB
A208	1	Ti Nop	††NT1(pTi-T37), Sciaky *et al.* 1977
K338	1	Ti Oct	C58C1(pTi-1001), This thesis
A281	Ti Ag	NT1	NT1(pTi-Bo542), Sciaky *et al.* 1977
R10	1	Ti Oct	Petit and Tempé 1978
K57A	1	Ti Nop	K57(pTi-27), *in planta* cross, A. Kerr
K57AAgr^{r}	1	"	agrocin 84 resistant mutant, P.J. Murphy, Adelaide
A623	1	"	A208, Agr^{r}, M.-D. Chilton
C58C1(pGV3181)	1	"	Holsters *et al.* 1980 Agr^{s}
C58C1(pGV3103)	1	"	" " " " Tn7 inserted into Agr^{s}
GV3804	1	"	C58C1CmEry(pTi-C58Tra^{c},::Tn7), J. Schell, Ghent
GV3575	1	"	C58C1(pTiT37α Nos^{-}::Tn7) J. Schell, Ghent
590	1	NOC	Kerr and Roberts, 1976

* nopaline Ti plasmid

** octopine Ti plasmid

*** agropine Ti plasmid

† nopaline catabolic plasmid (not a tumour inducing plasmid)

†† NTI is equivalent to C58C1 (Watson *et al.* 1975)

Names of well described strains (e.g. C58) are taken from the literature. New isolates are referred to by their number in Kerr's culture collection.

The bacterial cultures were stored at $4^{o}C$ on YM agar slopes and transferred every 6-12 months. For genetical experiments and plasmid isolations single colony isolations were made by streaking onto YM agar plates or onto plates that enabled direct selection for a particular phenotypic character of the strain. For example, when the experiment involved the nopaline Ti plasmid, the strain was purified through a single colony on nopaline medium.

B. MEDIA

The general rich growth medium was yeast mannitol agar (YM). It contained, per litre: K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.2g; NaCl, 0.2g; $CaCl_2$, 0.2g; $FeCl_3$, 0.01g; yeast extract, 1.0g; mannitol, 10g; Davis agar, 15g.

The minimal salt medium of Petit and Tempe (1978) was used. It contained, per litre: K_2HPO_4, 10.5g; KH_2PO_4, 4.5g; $MgSO_4.7H_2O$, 0.2g; $CaCl_2$, 10mg; $FeSO_4$, 5mg; $MnCl_2$, 2mg; biotin, 200μg; agar, separately sterilized, 20g. In most cases Difco-Bacto agar was used. Purified agar was also used. Minimal medium was supplemented with 2 mg/ml $(NH_4)_2SO_4$ and 1mg/ml mannitol.

Arginine, ornithine, nopaline, octopine and mannopine media consisted of Petit and Tempé's medium supplemented with one of these chemicals at 2mg/ml as sole sources of nitrogen and carbon. These chemicals were filter sterilized.

Stonier's medium contained per litre: $CaSO_4$, 100mg; $MgSO_4.7H_2O$, 200mg; NaCl, 200mg; NH_4NO_3, 2.7g; $Fe(NO_3)_3$, 5mg; $MnCl_2$, 0.1mg; $ZnCl_2$, 0.5mg; K citrate, 10g; Na glutamate, 2g; NaH_2PO_4, 0.3g; K_2HPO_4, 0.88g; biotin 200mg; agar, 15g; pH, 7.0.

The antibiotics in selective media were added from sterile solutions prior to pouring plates. The final concentrations used were: rifampicin 25µg/ml at first and later 100µg/ml; streptomycin, 500µg/ml; chloromycetin, 125µg/ml or 100µg/ml; erythromycin, 100µg/ml; spectinomycin, 200µg/ml. Stock solutions of rifampicin were made up at 10mg/ml in autoclaved dimethylsulphoxide (DMSO). Chloromycetin and erythromycin were also dissolved in sterile DMSO at 10mg/ml and 100mg/ml respectively. Aqueous solutions of antibiotics were filter sterilized. Solid rifampicin was sterilized by mixing it with a small volume of ethanol prior to dissolving it in DMSO.

C. SELECTION OF ANTIBIOTIC RESISTANT MUTANTS

Antibiotic resistant mutants that were used as recipients were isolated by plating 0.1ml of a $5x10^9$ cells/ml bacterial suspension onto minimal medium supplemented with the antibiotic.

D. TESTING GROWTH SUBSTRATES AS CARBON AND NITROGEN OR NITROGEN SOURCES

Single colony isolates of bacteria were suspended in 1ml of H_2O, vortexed to wash the cells and then streaked on the appropriate media. The bacteria were also streaked on the basic salt medium with no added nitrogen or carbon source to assess background growth and on minimal medium to determine whether the culture was viable and could grow

without extra growth factors. Where possible, control strains that were either able or unable to grow on the substrate under test were used as standards of growth and no growth.

E. AGROCIN 84 PRODUCTION AND SENSITIVITY TESTS

For agrocin 84 sensitivity tests the method of Kerr and Htay (1974) was used. An agrocin 84 producing strain was subcultured to a fresh YM slope and after two days this culture was used to inoculate plates of Stonier's medium. A loopful of the producer strain was applied as a 50mm spot in the centre of the plates and then these plates were incubated at 25°. At the same time the indicator strains were subcultured on YM slopes. After 2 days' growth the plates were sterilized for 15m with chloroform vapour. The indicator strain was then resuspended in 10ml of water and 0.5ml of the suspension added to 3ml of buffered soft agar at 45°C and poured onto the plate. When the soft agar layer had set, the plates were incubated at 27°C. A known sensitive and insensitive strain were included as controls.

When strains were being tested as agrocin producers the method above was also used. When many strains were being tested, a replica plating technique (Kerr and Panagopoulos, 1977) was used. The strains under test were spotted onto Stonier's agar (up to 20/plate) and after one day's growth were replica plated to a second plate of Stonier's medium. After one day's growth this replica was sterilized with chloroform vapour and overlayed with a sensitive indicator.

F. ISOLATION OF Noc^{c} AND Arc^{+} MUTANTS

Regulatory mutants of the nopaline catabolic system can be isolated by plating strains harbouring nopaline Ti plasmids onto octopine

medium (Petit and Tempé, 1978). Octopine is a non-inducing substrate for the nopaline catabolic system. Several types of regulatory mutants arise on this medium. One type is fully constitutive for nopaline catabolism and can be given the phenotypic designation Noc^c, whereas a second type has become inducible by octopine. Mutant nopaline strains able to use octopine have been used throughout the present study, and are referred to as Noc^c mutants. I wish to point out at this stage that this designation is not strictly correct since no formal proof has been given that the genes involved in nopaline catabolism are being expressed constitutively in these particular mutant strains. This would require the demonstration that each mutant used nopaline without a lag period and produced enzymes involved in nopaline catabolism without induction. This was not attempted. Nevertheless, the designation Noc^c has been used for convenience to describe the phenotype of nopaline catabolic regulatory mutants that have been selected for growth on octopine.

Noc^c and Arc^+ (mutants able to use arginine as sole carbon and nitrogen source) were isolated by plating a suspension of cells on octopine or arginine medium. When mutants of independent origin were required, a single colony growing on YM was suspended in 1ml of water and dilutions of this suspension were plated on either nopaline or arginine medium to give single colonies. Fast growing mutants appearing as sectors in individual colonies could only have arisen from independent mutations.

G. VIRULENCE TESTS

Virulence tests were carried out using an aqueous suspension of a 24-48h old culture of bacteria from YM agar slopes. One or two

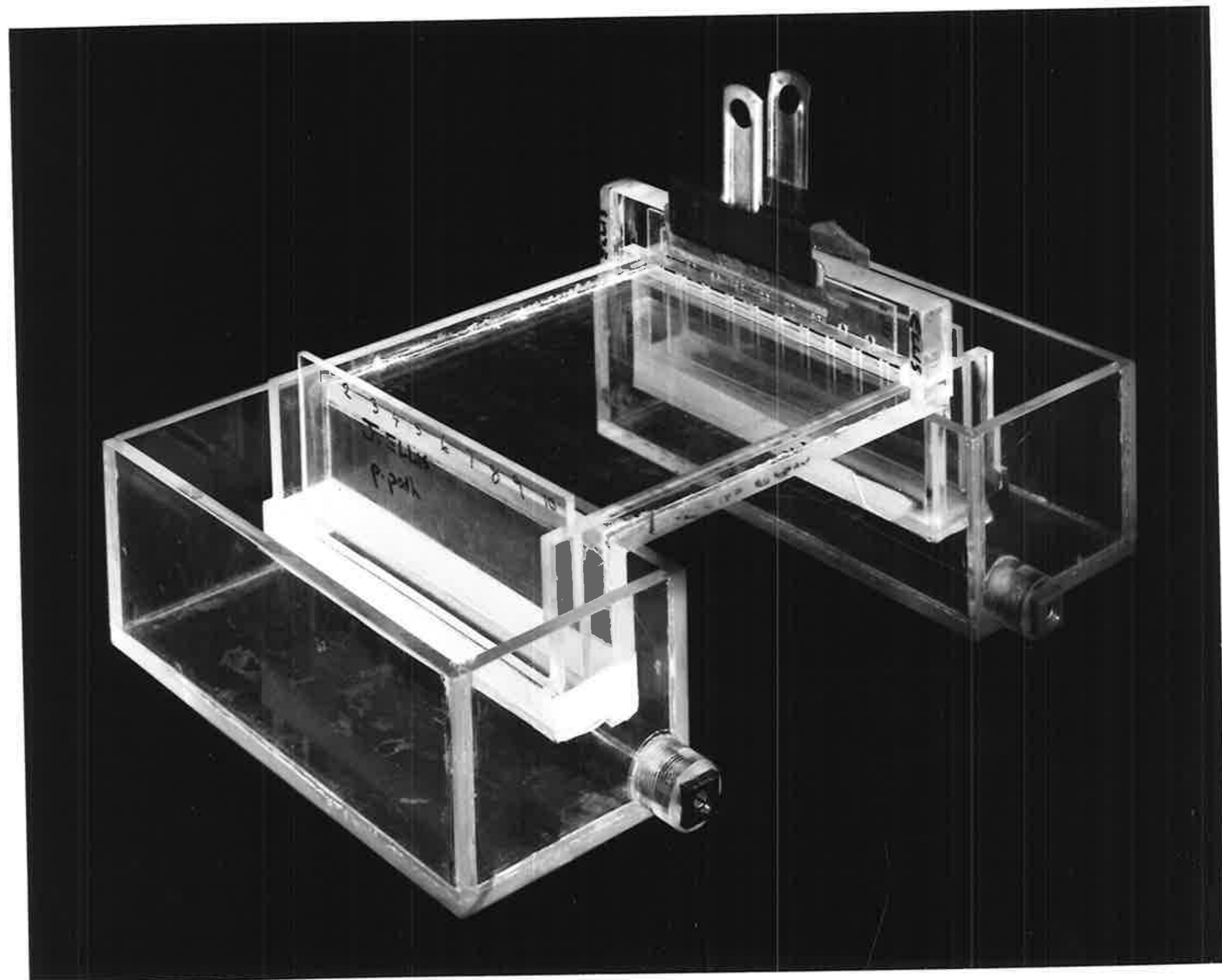
drops of the suspension were applied to the stems of 6-week old tomato plants. The stem was then wounded with a sterile needle. Alternatively, unsuspended bacteria were applied with a sterile loop and the stem wounded as above. When many strains were being tested, single colonies were inoculated onto tomato stems by stabbing the colony, then the stem with a sterile toothpick. Tumours appeared after 2-3 weeks.

H. DETECTION OF PLASMIDS BY AGAROSE GEL ELECTROPHORESIS

The following method was used. Fresh cultures of bacterial strains were made by transferring bacteria to YM slopes. After 1-2 days' growth, a loopful of bacteria taken from the YM slope and inoculated onto 25ml of PA (0.4% peptone and 2ml/l of 1M $MgSO_4$ solution) liquid medium in 125ml Ehrlenmeyer flasks. The cultures were shaken for approximately 16 hours and harvested when the bacteria reached about OD_{640} = 0.3. The bacterial cells were spun down at 10,000 rpm in a Sorval SS34 rotor for 10-15 minutes. The pellet was washed in 5ml of TE8 buffer (50mM Tris,20mM EDTA), then recentrifuged. The pellet was resuspended in 5ml of TE8 buffer and exactly 4ml of each culture was transferred to disposable 12ml capped tapered centrifuge tubes. The cells were then lysed by adding 0.5ml of protease solution (Sigma type VI, 5mg/ml TE8 self-digested at 37° for 60 min). Clear lysates resulted after 40-50 min at $37^{o}C$. DNA was denatured by adding sufficient 2N NaOH to raise the pH to 12.1-12.3. The amount of NaOH required was determined while the cells were lysing by measuring the volume required to raise the pH of a 'blank' tube consisting of 4ml of TE8 + 0.5ml of SDS solution + 0.5ml of protease solution. This avoided tedious pH

measurements of each lysate. This amount (about 180µl or 7 drops from a Pasteur pipette) was added to each lysate and mixed by several rapid inversions. Then, after gently inverting for 8 min the DNA was renatured by adding 300-350µl of 2M Tris-HCl, pH 7, to each tube and the lysates were again mixed by further gentle inversion for 2-3 min. (The amount of Tris-HCl added was also determined by adjusting the pH of the 'blank' to pH 8.5-9.0 while the bacteria were lysing.) Then 0.6ml of 5M NaCl was added and mixed by a single inversion. A precipitate appeared. The lysate was then extracted with 4.5ml of phenol equilibrated with 3% NaCl. (Reagent grade (Anal R) phenol can be used.) The phenol was prepared by melting the crystals at 60°C. An equal volume of sterile 3% NaCl was added and mixed by shaking. The phases were allowed to separate at 4°C overnight. (Pure phenol is clear and colourless.) After several rapid inversions to mix the phases, the contents of the tubes were mixed by gentle inversion for 7 min. The aqueous phase was separated by centrifugation and removed using the wide end of a 5ml pipette and safety bulb to avoid shearing. Pipettes whose diameter at the wide end was narrower than the body of the pipette were preferred, since the aqueous phase was less likely to drain out under gravity during transfer to sterile 50ml centrifuge tubes. The aqueous phase was adjusted to approximately 0.3M Na acetate (0.75ml of a 3M stock solution) and DNA was precipitated at -20°C overnight with two volumes of absolute ethanol. The precipitate was collected by centrifugation at 10,000 rpm for 10 min in a Sorval SS34 rotor, the supernatant was carefully poured off and the tubes were dried in the inverted position *in vacuo*. Inversion of the tubes caused less salt to precipitate with the DNA pellet. The dried precipitate was redissolved in 100µl of TES 8 buffer (50mM Tris, 50mM NaCl and 5mM EDTA)

Figure 2-1. Horizontal slab gel apparatus based on the design of McDonell *et al.* (1977). Dimensions were 180mm long and 140mm wide. The well former teeth were 8mm in width and cut from 2mm thickness perspex. The teeth were 3mm apart. Gels were poured to a thickness of 6mm.



and 40 l of tracking dye (20% ficoll, 0.1% SDS, 0.02% bromophenol blue). Samples of 20 to 40µl were electrophoresed in 0.7% Seakem agarose in Tris-borate buffer (Meyers *et al*., 1978) at 150V (approximately 1 V/cm length of gel). Horizontal slab gels were used (Figure 2-1). Electrophoresis was stopped when the blue tracking dye had travelled at least 16 cm. DNA was stained with 0.5µg/ml ethidium bromide solution in Tris-borate running buffer for 30 min. Gels were photographed under short wavelength UV light using a black porcelain tile as background. Ilford Pan F film and a yellow filter were used. The aperture was set at F2.8 and several exposures were made from 5-15 min. The film was developed and the best negative printed. This print was mounted, the tracks labelled, then rephotographed professionally.

I. PLASMID DESIGNATION

Agrobacterium plasmids have been designated following Sciaky *et al*. (1977). Wild type Ti plasmids are designated pTi-X where X is the strain name used in the literature. For example, the Ti plasmid of strain T37 is designated pTi-T37. Other plasmids of *Agrobacterium* are designated At-Xa, At-Xb, etc., where X is the strain name and a, b, etc., are used to designate different plasmids in the strain. Plasmids in strains supplied by Professor J. Schell are designated by the Ghent number which is in accord with the recommendations of Novick *et al*. (1976). This nomenclature was also used for the series of Tra^{C} mutants of pTi-C58, described in Chapter 8.

J. TRANSCONJUGANT DESIGNATIONS

Where the plasmid content of a transconjugant has been determined,

transconjugant strains are designated by the strain number of the recipient plus the plasmid(s) derived from the donor (e.g. C58Cl(At-84b).) Where the plasmid content has not been determined, the transconjugant is designated by the cross from which it resulted and a number. The characters inherited from the donor are indicated in the text. For example, (590Noc^{c} x 398RifStr)#1 is a transconjugant resulting from a cross between donor strain 590Noc^{c} and recipient strain 398RifStr. The donor is given as the first strain in the cross.

K. EXPERIMENTS INVOLVING [^{32}P]AGROCIN 84

[^{32}P]agrocin 84 was prepared by P.J. Murphy using the methods described by Murphy and Roberts (1979). For uptake studies, cultures were grown overnight in Stonier's medium, centrifuged and resuspended in the same medium. After addition of [^{32}P]agrocin 84, samples (2 x 50μl) were taken at times indicated in the relevant figures, filtered through Millipore membranes (0.45μm pore size) and washed with Stonier's medium. The filters were dried in a scintillation vial at 110° for 10 min. Scintillant [2ml, containing dimethyl-POPOP, (0.3g/l) and PPO (8g/l)] was added to each tube and the radioactivity counted in a Packard Tricarb Scintillation Counter.

L. PHAGE TYPING OF BACTERIAL STRAINS

Phage typing was carried out to confirm that strains were transconjugants. Putative transconjugants, the donor and the recipient strains were tested simultaneously. The phage types of strains used regularly as recipients and donors are shown in Table 2-2.

The bacteriophage S1, S2, S3 and S5 were supplied by J. Schell, Ghent, and Ø3, Ø117, Ø124 and Ø156 were isolated from sewage in Adelaide by Dr S.M. Hocking.

Phage were stocked either by making liquid infections of host bacteria in early log phase growing in YM liquid medium or by making plate stocks. Plate stocks were made by flooding plates that had been overlayed with a host strain plus a dilution of phage that was just sufficient for confluent lysis. Plates were flooded with 3ml of nutrient broth. The nutrient broth was removed after 24h at $4^{o}C$ and sterilized by filtration.

Phage typing was done by spotting approximately 20µl of a phage stock (10^{6} - 10^{7}pfu/ml) onto a plate of YM agar freshly poured with a soft agar overlay containing 0.5ml of a bacterial cell suspension containing 10^{9} cells/ml.

Table 2-2. Phagetypes of strains used regularly in this thesis*.

	S1	S2	S3	S5	Ø3	Ø117	Ø124	Ø156
C58	-	+	-	+	+	-	-	NT
C58C1	-	+	-	+	+	-	-	NT
K230	-	+	-	+	+	-	-	NT
K264C1	-	+	-	+	+	-	-	NT
K57	+ or - variable	-	-	-	+	+	-	NT
B9105	+	-	+	-	+	NT	NT	NT
NCPPB1001	+	+	-	-	-	+	+	NT
R10	+	+	-	-	-	+	+	NT
Bo542	-	+	-	-	-	+	+	NT
NCPPB398	-	-	-	-	-	-	-	+
T37	-	-	-	-	+	+	+	NT

+ = lysis   - = no lysis   NT = Not Tested

* None of these phage lysed biovar 2 or 3 strains of *Agrobacterium radiobacter*.

CHAPTER 3

ARGININE CATABOLISM - A FUNCTION COMMON TO NOPALINE AND OCTOPINE Ti PLASMIDS

INTRODUCTION

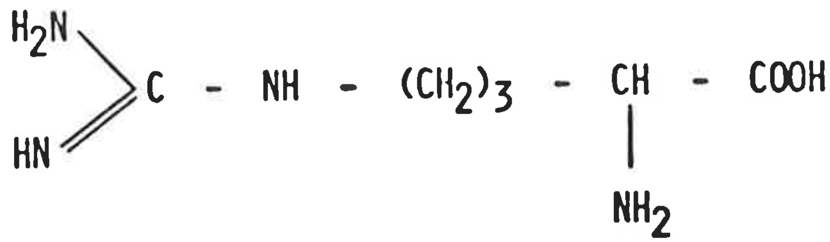
The ability to degrade either of the arginine derivatives, octopine [N^2-(1-carboxyethyl)arginine] or nopaline [N^2-(1,3)-dicarboxy-propyl - arginine] characterises two classes of Ti plasmids, the octopine and nopaline Ti plasmids (Bomhoff *et al.*, 1976; Chilton *et al.*, 1976). Petit *et al.* (1970) have shown that octopine catabolism proceeds via arginine. From its formula (Figure 3-1) it is likely that nopaline is also degraded via arginine. Is arginine also degraded by Ti plasmid encoded pathways? This possibility was examined and evidence is presented in this chapter that both octopine and nopaline Ti plasmids are involved in degradation of arginine to supply a source of carbon to *A. radiobacter*.

EXPERIMENTAL AND RESULTS

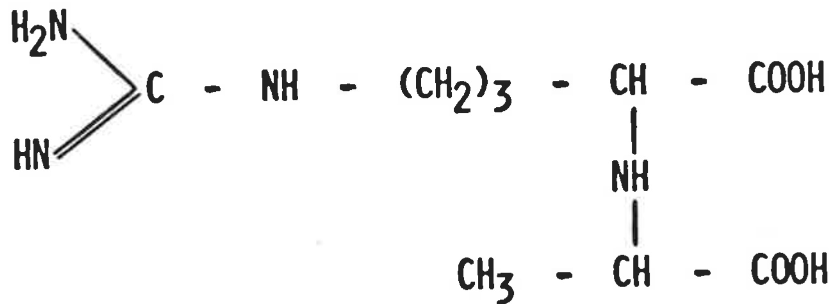
Section A. Strain C58 and its derivatives

During the testing of various strains for growth on solid medium containing 2 mg/ml arginine as sole source of carbon and nitrogen (referred to as arginine medium) strain C58 and its Ti plasmidless derivative C58CI did not grow. However, when strain C58 which harbours a nopaline Ti plasmid was plated on arginine medium

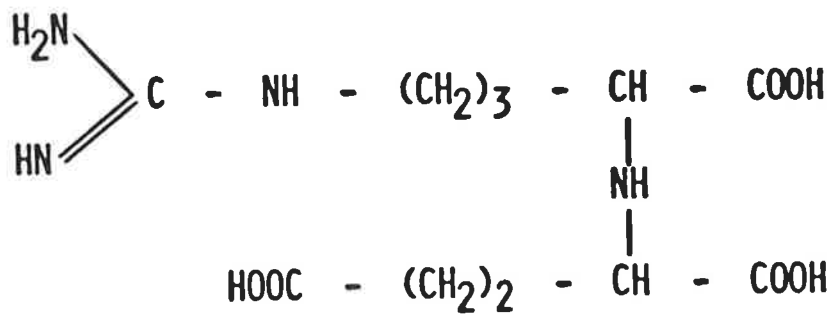
Figure 3-1. Structures of arginine and its derivatives octopine and nopaline.



ARGININE



OCTOPINE



NOPALINE

mutant colonies appeared that used arginine. The phenotype of these mutants is designated Arc$^+$. These mutants arose quite frequently. In contrast, when strain C58CI was plated on arginine medium, mutant colonies arose very rarely. Since C58 and C58CI differed only in the presence of the plasmid pTi-C58, the possibility was considered that the Arc$^+$ phenotype of the C58 mutant was due to a mutation on the Ti plasmid. Independent C58Arc$^+$ mutants were isolated by plating a dilution of a culture of C58 to give single colonies on arginine medium. These colonies grew very slowly on contaminating carbon sources in the agar but after 2 to 3 weeks incubation at 27°, mutant sectors of these colonies appeared that grew rapidly on arginine medium. Five mutants were purified by restreaking an arginine medium. These mutants were tested for growth on octopine medium (2 mg/ml octopine as sole source of carbon and nitrogen). All grew rapidly on octopine medium, whereas the parent strain C58 did not. A single C58CIArc$^+$ mutant was also tested and did not grow on octopine medium.

Octopine is a non-inducing substrate of the nopaline catabolic system and when nopaline strains are plated on octopine medium mutants arise that grow rapidly on this substrate. These are regulatory mutants of the nopaline catabolic genes (Petit and Tempé, 1975, 1978). . The C58Arc$^+$ mutants also grew on octopine medium, Therefore these mutants behaved as regulatory mutants of the nopaline catabolic system. Regulation of this system is controlled by Ti plasmid linked genes (Kerr *et al.*, 1977; Holsters *et al.*, 1978).

Another nopaline strain K344, which is a Ti plasmid transconjugant strain resulting from a cross between donor strain T37 and recipient

strain C58ClRifStr, was examined for arginine catabolism. The transconjugant was selected for growth on nopaline. [After this series of experiments had been completed this strain was found to contain pTi-T37 and also a cryptic plasmid smaller than the Ti plasmid which had also transferred from T37 (results not shown).] K344 was plated on arginine medium. Several Arc$^+$ mutants (whose independent origin cannot be guaranteed from the method of their isolation) were purified and tested for growth on octopine medium. All the mutants used octopine as sole source of carbon and nitrogen. Strain K344 did not. These Arc$^+$ mutants also behaved as regulatory mutants of the Ti plasmid linked nopaline catabolic system.

Conjugation experiments have confirmed that the nopaline Ti plasmid carries the genes that enable C58CI to grow on arginine. A nopaline catabolic regulatory mutant of strain T37 that grew on octopine medium was selected. This mutant, T37-3/1, was used as donor in a cross with C58CIRifStr. The donor was patched onto an agar plate containing 0.2mg/ml octopine. The recipient was spread on a separate plate containing the same medium. After 24h growth, the donor patches were replica plated onto the recipient and after a further 24h this plate was replica plated to arginine medium containing rifampicin (25μg/ml) and streptomycin (500μg/ml). Arc$^+$ transconjugants appeared and ten independent transconjugants were purified, one from each of 10 separate patches of transconjugants on the selective plate. All the transconjugants grew on octopine medium, were pathogenic on tomato seedlings and had the phage-type of C58CI. Therefore, by selecting for arginine catabolism, transconjugants harbouring the Ti plasmid were

selected. Similarly, Ti plasmid transconjugants of C58CIRifStr containing a $Tra^{c}Noc^{c}$ derivative of pTi-C58 have been selected on arginine medium.

Further evidence that the Ti plasmid is involved in arginine catabolism came from experiments in which strains were cured of pTi-C58. In one experiment a Noc^{c} mutant strain, C58CI(pWI1009)(pAt-84a) (see Chapter 5), was grown at 37° for 3 days, then plated for single colonies on octopine medium. The parental strain grew on octopine and arginine medium. After growth at 37°, few colonies grew on octopine medium. A single colony that did not utilise octopine was chosen for further study. It did not grow on arginine medium and had lost the Ti plasmid (see Chapter 5).

In a second experiment mutants of an Arc^{+} derivative of strain C58 that were resistant to agrocin 84 were isolated by suspending in water the soft agar layer from within the inhibition zone of an agrocin 84 bioassay plate (Kerr and Htay, 1974) and then plating dilutions onto arginine medium. Less than 1% of the agrocin 84 resistant mutants grew on arginine. Engler *et al.* (1975) have shown that 5 out of 5 independent agrocin 84 resistant mutants of C58 had lost the Ti plasmid and have shown that sensitivity of agrocin 84 is linked to the Ti plasmid. By inference, the loss of the Arc^{+} phenotype by the agrocin 84 resistant mutants of the $C58Arc^{+}$ mutants is probably due to loss of the Ti plasmid.

Arginine catabolism and the octopine Ti plasmids

A transconjugant strain C58CI(pTi-1001) which harbours an octopine Ti plasmid was plated on arginine medium and arginine utilizing mutants arose. One mutant, C58CI(pTi-1001)Arc+ was studied in more detail.

Thus mutant also utilized histopine (Kemp, 1977) as sole source of carbon and nitrogen. Histopine is a non-inducing substrate of the octopine catabolic system (Petit and Tempe, 1978). This mutant, therefore has the properties of a regulatory mutant of the octopine catabolic system. These observations have been confirmed and extended showing that arginine catabolism is also a property of octopine Ti plasmids and that arginine catabolism results from constitutive octopine catabolism (Petit *et al.*, 1978b; Ellis *et al.*, 1979a).

Octopine and nopaline induce Ti plasmid linked arginine catabolism

Since octopine and nopaline induce their own catabolic genes (Petit and Tempe, 1978) and regulatory mutants of these catabolic systems give rise to the Arc^{+} phenotype, it follows that these opines must also induce the synthesis of enzymes involved in Ti plasmid-linked arginine catabolism. The following experiment was carried out to test the effect of octopine and nopaline on arginine catabolism. C58CI harbouring the wild type nopaline Ti plasmid of T37 was plated on minimal medium containing 10mM arginine as sole source of nitrogen and carbon, on 9mM plus 1mM nopaline (to induce nopaline catabolism) and on 1mM nopaline alone as a control. Very thin growth occurred on 1mM nopaline alone due to the low substrate concentration; virtually no growth occurred on arginine alone and good growth occurred on 1mM nopaline plus 9mM arginine. The results indicate that nopaline induces arginine catabolism in C58CI(pTi-T37). A similar experiment was carried out using a transconjugant harbouring a wild type octopine Ti plasmid from strain R10. It was found that 1mM octopine induced arginine catabolism allowing the transconjugants to use arginine as a source of carbon and nitrogen. The results are

Table 3-1.

Strain	Culture medium				
	10mM arg	9mM arg +1mM oct	9mM arg +1mM nop	1mM oct	1mM nop
C58C1	-	-	-	-	-
C58C1(pTi37)	-	-	+++	-	+
C58C1(pTiR10)	-	+++	-	+	-
C58C1(pTiT37Noc^{c})	+++	+++	+++	+	+
C58C1(pTiR10Occ^{c})	+++	+++	+++	+	-

Growth of C58C1 and its derivatives on arginine with and without induction by opines.

Abbreviations and symbols:

arg = arginine, oct = octopine, nop = nopaline,

+ = thin growth, +++ = full growth, - = no growth.

presented in Table 3-1. Therefore, the arginine catabolic activity of octopine and nopaline Ti plasmids is induced by the respective opine and not by arginine itself.

Section B. Strain T37 and Its Derivatives

The nopaline strain T37 maintained in Adelaide does not grow on arginine as sole source of carbon and nitrogen. It was supplied by G. Morel of Versailles. Curiously, the culture of T37 maintained in Versailles can grow on arginine medium (J. Tempé, personal communication). The Adelaide strain is described below.

Strain T37 differs from strain C58 in several ways with respect to arginine catabolism. First, although T37 cannot grow on arginine medium, it can grow on ornithine medium (2mg/ml ornithine as sole source of carbon and nitrogen). Strain T37 mutates very readily to Arc^+ but unlike C58Arc^+ mutants, very few T37Arc^+ mutants are able to grow on octopine as sole source of carbon and nitrogen (octopine medium). For example, in one experiment 1.5×10^8 cells of strain T37 were plated onto a plate of octopine medium and a plate of arginine medium. After 10 days, 62 Arc^+ mutants arose on arginine medium, while only 5 mutants arose on the octopine plate. When the Arc^+ mutants were replica plated onto octopine medium, 11 octopine using patches appeared corresponding to Arc^+ colonies on the master plate.

Two independent T37Arc^+ mutants that did not grow on octopine medium were used as Ti plasmid donors to C58CIRifStr. Transconjugants from each cross were selected for growth on nopaline medium, purified on nopaline medium and then tested for growth on arginine medium. The transconjugants did not grow. It was concluded that the Arc^+ phenotype of these mutants was not associated with the Ti plasmid.

When mutants of T37 were selected for growth on octopine medium, two classes of mutants could be distinguished by their growth characteristics on arginine medium. One group grew on arginine medium and the other did not. The first group also utilised octopine faster than the second (A. Kerr, personal communication). It has been suggested (J. Tempé, personal communication) that the second group contains regulatory mutants of the nopaline catabolic system that have become inducible by octopine (Petit and Tempé, 1978).

Strain T37-3/1 is an example of the group of mutants that grow on both octopine medium and on arginine medium. As described above in Section A, Ti plasmid transconjugants can be selected for growth on arginine selective medium when T37-3/1 is used as donor and C58CIRifStr is used as recipient, which indicates that the ability to catabolise arginine in this mutant is controlled by the Ti plasmid.

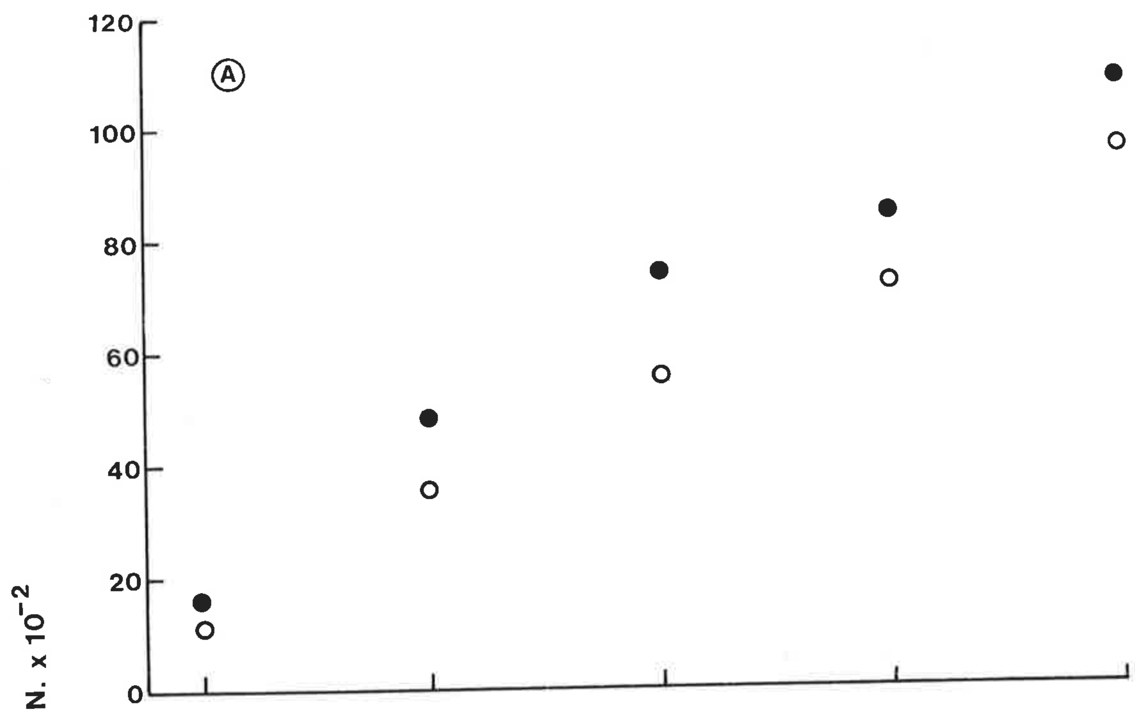
Section C. The Mechanism and Pathway of Ti Plamid-Linked Arginine Catabolism

One mechanism that was considered to account for the involvement of the Ti plasmids in arginine catabolism was that although strain C58 and T37 may possess genes necessary for growth on arginine as carbon source, their arginine permease may not be efficient; this deficiency may be supplemented by a Ti plasmid-linked permease that can take up arginine. This hypothesis was tested by comparing the uptake of [U-^{14}C]-arginine by C58CI(Arc^{-}) and C58CI(pTi-T37-3/1)(Arc^{+}) and also T37(Arc^{-}) and T37-3/1(Arc^{+}). The method used was based on that

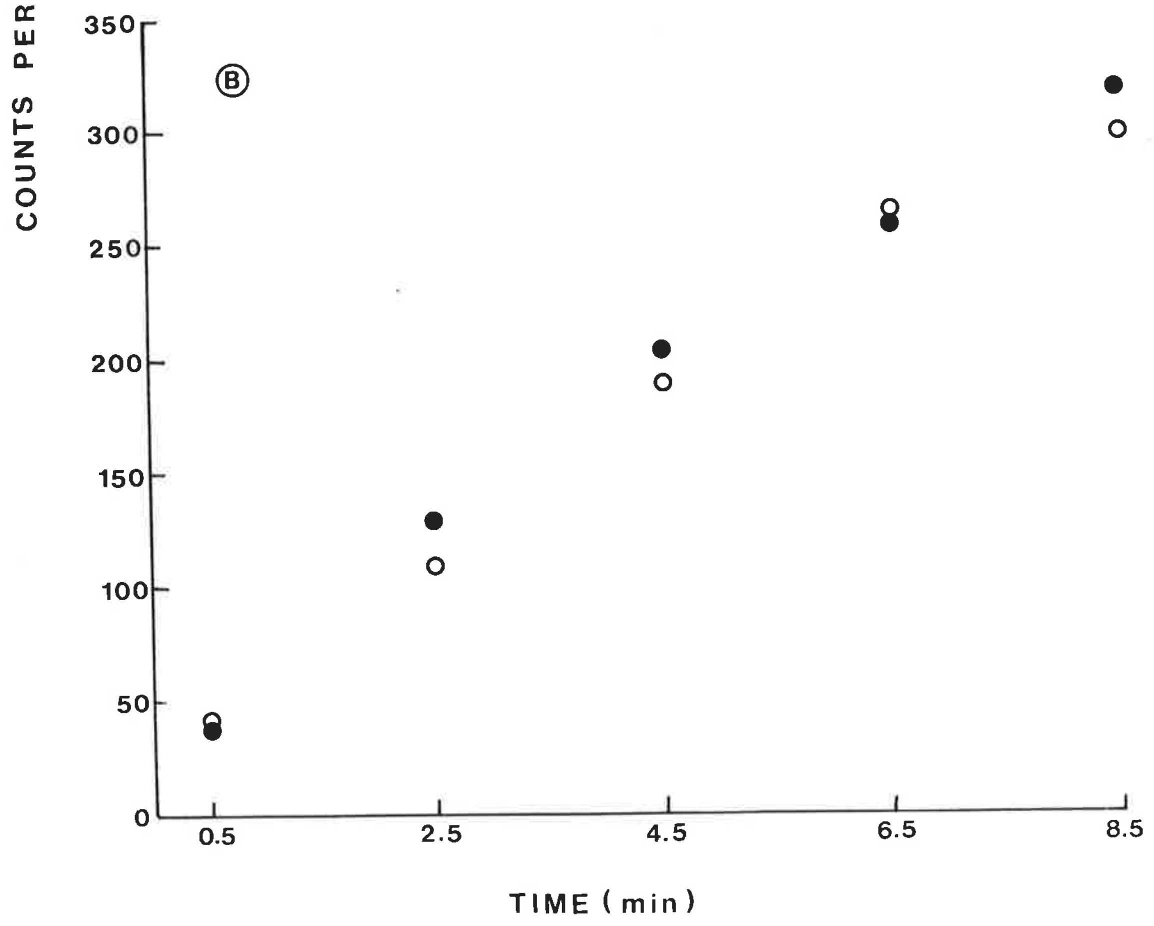
described by Miller and Rodwell (1971). The strains were grown for 2 days on agar slopes of the minimal salts medium of Petit and Tempe (1978) containing $(NH_4)_2SO_4$ (2mg/ml) and glucose (2.5mg/ml). The bacteria were then suspended on the same mineral medium (without $(NH_4)_2SO_4$ and glucose), washed twice, resuspended to give an OD of 6 mesured using an Eel Absorbtiometer (approximately 10^8 cells/ml) and kept in an ice bucket until needed. Ten µl of $[^{14}C]$arginine solution (50µCi/ml) was added to a tube and then 2 ml of bacterial suspension that had been preincubated at 25° for 10 min was added. After 30 seconds incubation at 25°, a 250µl sample was removed and filtered through a 0.45 Millipore filter. The filter was washed twice with 5ml of buffer and then placed immediately into dioxan fluor. Further samples were taken at 2.5, 4.5, 6.5 and 8.5 min. Samples were counted on a Packard Tricarb scintillation counter for 10 min. Two replicates of each strain were done and the average values calculated. The results are presented in Figure 3-2a and 3-2b. No major difference could be detected between isogenic strains that differed in their ability to grow on arginine medium. It was concluded that the arginine utilisation phenotype does not depend on increased arginine permeation encoded by the Ti plasmid.

Since a Ti plasmid-linked permease is not involved in arginine catabolism the Ti plasmids probably encode enzymes involved in arginine catabolism. No enzymological studies were carried out but a preliminary investigation to define the pathway of arginine catabolism was begun. Ornithine and citrulline were tested as nitrogen and as carbon and nitrogen sources by streaking suspensions of various strains on media containing these amino acids. The results are presented in Table 3-2.

Figure 3-2. Uptake of [U-^{14}C] arginine by A, T37 ● ● and T37-3/1 o o and B, C58C1 ● ● and C58C1(pTi-T37-3/1) o o.



120
100
80
60
40
20
0
A
COUNTS PER MIN. x 10^{-2}



350
300
250
200
150
100
50
0
B
0.5
2.5
4.5
6.5
8.5
TIME (min)

Table 3-2.

Strain	Culture medium			
	1mM ornithine +9mM mannitol	1mM citrulline +9mM mannitol	10mM ornithine	10mM citrulline
C58Cl	+	+	-	-
C58Cl(pTiT37Noc^{c})	+	+	+	-
C58Cl(pTi1001Occ^{c})	+	+	+	-

Growth of C58Cl and its derivatives on ornithine and citrulline as sole nitrogen and carbon source + = full growth, - = no growth.

The C58CI transconjugants harbouring either an octopine or nopaline Ti plasmid that carried an opine catabolic regulatory mutation were the only strains that grew on ornithine as sole source of C and N. None grew on citrulline as sole source of carbon and nitrogen. All strains used these amino acids as source of nitrogen. Ornithine but not citrulline is therefore a likely intermediate in arginine degradation. Since C58CI is able to use arginine and ornithine as source of nitrogen, the block in the degradative pathway of arginine in the strains must involve the release of a carbon source from arginine.

Strain T37 however, which is unable to use arginine as a source of carbon, is able to use ornithine as a carbon source. Conjugation experiments showed that the ability of this strain to grow on ornithine was not conferred on the recipient strain C58CI by the wild type pTi-T37. So the enzymes involved in ornithine catabolism by T37 are either encoded by the chromosome or another plasmid. As shown above however, the regulatory mutant plasmid pTiT37-3/1 does confer the ability to catabolise ornithine in strain C58CI. Therefore, there must be two sets of genes in T37 that are involved in ornithine catabolism. One set linked to the Ti plasmid and part of the nopaline catabolic system is not inducible by ornithine and the second set found elsewhere in the genome are presumably inducible by ornithine.

Since T37 can use ornithine as source of carbon, the block in the degradative pathway of arginine in this strain is at a different point from that in strain C58CI. It is possibly at a step where arginine is converted to ornithine. Since the Ti plasmid-linked regulatory mutant in T37-3/1 allows this strain to use arginine as source of carbon, it is possible that arginine is converted to ornithine

by a Ti plasmid encoded enzyme, for example arginase. This possibility was not further investigated.

DISCUSSION

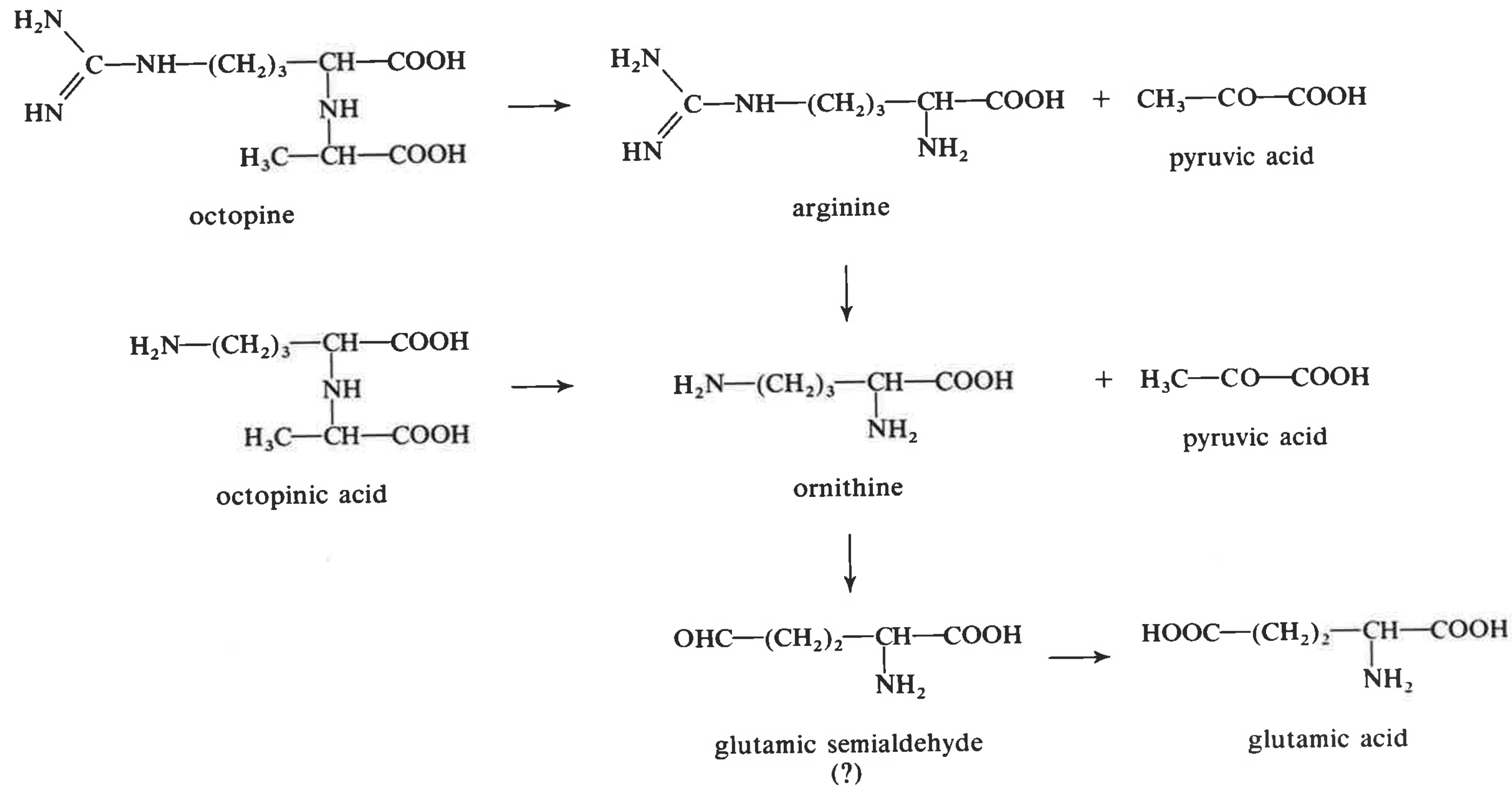
The results presented in this chapter show that octopine and nopaline Ti-plasmids are involved in the catabolism of arginine, or more precisely, of an arginine degradation product that can provide a carbon source. The results rely on having the strain C58CI that is cured of its Ti plasmid and that does not grow on arginine or ornithine as a carbon source. When a Ti plasmid that is induced or constitutive for opine catabolism is present in this strain, it is then able to grow on arginine or ornithine as a carbon source. The opines nopaline and octopine, are arginine condensation products. The octopine and nopaline Ti plasmids code for at least 3 steps involved in the catabolism of these opines. The first is an opine permease (Klapwijk *et al.*, 1977), the second of these cleaves the opine into arginine and an α-ketoacid, and the third degrades an arginine derivative to a compound that can be used as a carbon source by the cell. It is likely that this compound is glutamic acid, since Petit *et al.* (1970) have shown that when an octopine strain is fed [^{14}C]-octopine labelled in the arginine part of the molecule, the largest fraction of the label is recovered as glutamic acid. Glutamic acid can be used as sole carbon source by strain C58CI.

The pathway of octopine and nopaline catabolism appears to proceed via arginine and ornithine to glutamic acid. Evidence that arginine is a product of octopine degradation comes from the work of Petit *et al.* (1970) and also from the work of Chilton *et al.* (1976).

The latter authors showed that octopine and nopaline can satisfy arginine auxotrophy in strains of *A. radiobacter* carrying an octopine or a nopaline Ti plasmid. Furthermore, it has been shown that an arginine auxotroph of C58CI, whose deficiency cannot be satisfied by ornithine, can also grow with a supplement of octopine or nopaline when it carries an octopine or a nopaline Ti plasmid (Hernalsteens and Ellis, unpublished results). Figure 3-3 shows the pathway proposed by Ellis *et al.* (1979a) for octopine catabolism. The results of Wu and Unger (personal communication from L. Unger) have led them to propose the same pathway.

The steps involved in making nitrogen available to the cells occur in the Ti plasmid-cured strain C58CI, so I am as yet unable to say whether these steps may also be specified by the Ti plasmids. Since C58CI is able to utilise both arginine and ornithine as nitrogen source and glutamic acid as source of both carbon and nitrogen, the block in the degradation of arginine must occur prior to glutamic acid and may involve the conversion of glutamic semialdehyde (GSA) to glutmaic acid. However GSA, or its cyclised form Δ-1,2 pyrrolline 5-carboxylic acid, is also an intermediate in the degradation of proline in several species of bacteria (De Hauwer *et al.*, 1964; Dendinger and Brill, 1970). Strain C58CI can grow on proline as carbon and nitrogen source, yet not on arginine, even in the presence of 1mM proline to induce the proline degradative pathway (unpublished results). This may argue against the involvement of GSA in the Ti plasmid-coded arginine degradation. Another pathway of arginine degradation, via agmatine and putrescine, has been reported to occur in *A. radiobacter* (Speranza and Bagni, 1977). However this pathway is unlikely to operate here, if, as may be inferred from the results of Petit *et al.* (1970), glutamic acid is the product

Figure 3-3. Pathway of catabolism of octopine and octopinic acid in C58Cl(pTi octopine) as proposed by Ellis *et al.* (1979a). The steps indicated by horizontal arrows make carbon available and are controlled by Ti plasmid genes. The steps indicated by vertical arrows make nitrogen available.



Proposed pathway of octopine and octopinic acid catabolism in C58Cl (pTi oct). The steps indicated by horizontal arrows make carbon available and are controlled by Ti-plasmid genes. The steps indicated by vertical arrows make nitrogen available

of arginine degradation.

The results of Hernalsteens (1978) and Holsters *et al.* (1980) confirm that the catabolism of arginine as carbon source is controlled by the Ti plasmids. The former author isolated transposons in an octopine Ti plasmid that eliminated arginine catabolism of a C58CI transconjugant strain. The latter authors have isolated transposon insertions in the nopaline Ti plasmid pTiC58 that have the same effect. Moreover, the results of Holsters *et al.* (1980) also imply that arginine catabolism and nopaline catabolism are controlled by genes in a single operon.

From these results, it is clear that the expression of the plasmid borne gene(s) involved in arginine degradation is not dependent on the presence of arginine, but on the presence of an opine which is both the inducer of opine and arginine catabolic functions, and also the source of arginine. Petit and her coworkers (Petit and Tempe, 1978; Petit *et al.*, 1978b) have described mutants of an octopine transconjugant of strain C58CI that have lost the ability to utilise octopine as a carbon source. Some of these mutants behave like typical polar mutants. They have simultaneously lost the ability to grow on octopine, arginine and ornithine as carbon source and give true revertants on any of these substrates. These mutants may carry a polar non-sense mutation in the structural gene coding for the cleavage of octopine and imply that the genes involved in the catabolism of octopine and arginine belong to the same operon.

CHAPTER 4

TRANSFER OF ARGININE CATABOLIC ABILITY WITHOUT Ti PLASMID TRANSFER

INTRODUCTION

Unlike C58 and its derivatives, most strains of *A. radiobacter* that have been tested are able to grow on arginine medium. The genetic basis of arginine catabolism in one strain, NCPPB1001, was studied. This strain is pathogenic and harbours an octopine Ti plasmid. Before the genetics of the regulation of Ti plasmid encoded arginine catabolism had been fully elucidated, the following hypothesis (which was subsequently disproved) was considered. It was proposed that the ability of strain NCPPB1001 to grow on arginine medium was due to expression of Ti plasmid genes. To test this hypothesis, pTi-1001 was transferred by conjugation to strain C58CIRifStr which cannot grow on arginine medium. It was argued that if arginine catabolism was due to the expression of genes on the Ti plasmid it should be possible to select Ti plasmid transconjugants on a selective medium containing arginine as sole source of carbon and nitrogen. This experiment was attempted and transconjugants did appear on arginine selective medium but in many cases the transfer of the ability to grow on arginine medium was not accompanied by the transfer of the Ti plasmid markers, virulence and octopine catabolism. The genetic basis of the transfer of arginine catabolism in this experiment is the subject of this chapter.

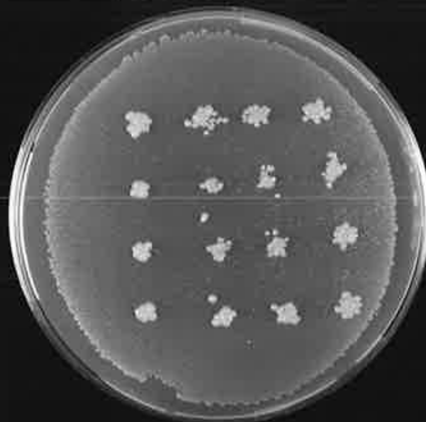
EXPERIMENTAL AND RESULTS

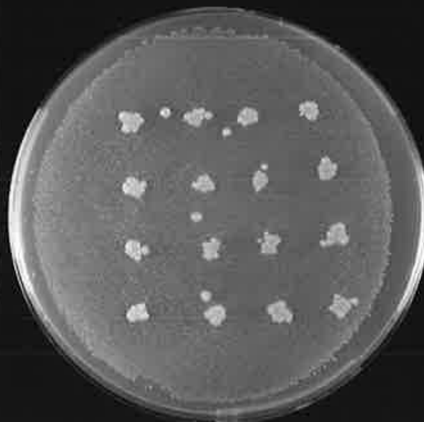
A genetic cross was made between NCPPB1001 (donor) and strain C58CIRifStr (recipient). Strain 1001 was patched onto a master plate containing 2mg/ml arginine. At the same time, C58CIRifStr (which cannot use arginine or octopine as sole source of carbon) was spread on 'conjugation' plates that contained either 0.2mg/ml octopine or 0.2mg/ml arginine. The plates were incubated for 24 h then the donor strain was replica plated onto the recipient lawn growing on the 'conjugation' plates. After a further day's growth, the bacteria on these plates were replica plated onto selective media containing 25μg/ml rifampicin and 500μg/ml streptomycin to counterselect the donor and either 2mg/ml octopine or arginine to select for the ability to use either of these compounds as carbon and nitrogen sources. The results are shown in Figure 4-1. After conjugation in the presence of octopine, transconjugants arose on both octopine and arginine selective media. No transconjugants arose after conjugation on arginine. Clearly transfer of both the ability to use arginine and the ability to use octopine had been promoted by octopine.

The octopine-using transconjugants were Ti plasmid transconjugants. As explained in Chapter 3, C58CI(pTi-1001) does not grow on arginine, therefore the basis of the transfer of the ability to use arginine was investigated. One colony from each patch of transconjugants on arginine selective media was purified on arginine medium to give 14 independently derived transconjugants. Similarly, independent transconjugants from octopine selective medium were purified on octopine medium. Each

Figure 4-1. Transconjugants from the cross 1001 x C58ClRifStr on octopine and arginine selective media after conjugation on octopine or arginine medium.

conjugation medium
octopine
arginine
octopine
selection
medium
arginine





transconjugant was cross-checked for growth on arginine and on octopine media, tested for virulence and phage typed. None of the transconjugants from octopine selective plates grew on arginine medium. Four out of 14 transconjugants from arginine selective medium grew on octopine medium. These were also virulent on tomatoes indicating they had inherited the Ti plasmid. In the other transconjugants the Ti plasmid was not transferred. Arginine catabolism in these transconjugant strains was not linked to the Ti plasmid. Six of these arginine users were tested for growth on ornithine as sole source of carbon and nitrogen and all grew.

The donor, recipient and transconjugants were tested for phagetype. A mutant of the recipient C58CI that was selected for growth on arginine medium and a mutant of a transconjugant C58CI(pTi-1001) that could grow on arginine medium was also tested. The latter strain used arginine via the derepressed Ti plasmid-linked octopine catabolic system (see Chapter 3). Several strains were tested for growth on histopine (2mg/ml) as source of carbon and nitrogen. The strains tested and their phenotypes are shown in Table 4-1. It is interesting to note that many of the transconjugants from arginine selective plates were resistant to the bacteriophage S5, which is highly specific for C58 and its derivatives. Towo arginine-using transconjugants were sensitive. This latter class could not be distinguished from the rare mutants of C58CI that arise on arginine medium. It is unlikely, however, that these transconjugants are mutants because no arginine-using strains appeared in the control when the donor strain had not been precultured on octopine. The phenotype of the remaining strains which are sensitive to phage which lyse strain C58CI but not 1001 leaves no doubt that the

Table 4-1. The Phenotypes of Strains 1001, C58CI and its Derivatives.

Strain	Arc[a]	Occ[b]	Hic[c]	Virulence	Phage Type						
					S1	S2	S3	S5	Ø3	Ø117	Ø124
C58CI	-	-	-	-	-	+	-	+	+	-	-
1001	+	+	-	+	+	+	-	-	-	+	+
C58CIArc+	+	-	-	-	-	+	-	+	+	-	-
*exoct1	-	+	-	+	-	+	-	+	+	-	-
≁exoct1Arc+	+	+	+	+	-	+	-	+	+	-	-
exoct2	-	+	NT	+	-	+	-	+	+	NT	NT
≠exarg1	+	-	-	-	-	+	-	-	+	-	-
2	+	-	NT	-	-	+	-	-	+	NT	NT
3	+	-	-	-	-	+	-	+	+	-	-
4	+	+	NT	+	-	+	-	-	+	-	-
7	+	+	NT	+	-	+	-	-	+	NT	NT
9	+	-	NT	-	-	+	-	-	+	NT	NT
10	+	-	NT	-	-	+	-	-	+	NT	NT
11	+	-	NT	-	-	+	-	-	+	NT	NT
12	+	-	NT	-	-	+	-	-	+	NT	NT
13	+	+	-	+	-	+	-	+	+	-	-
14	+	+	-	+	-	+	-	-	+	NT	NT

a growth on arginine medium

b growth on octopine medium

c growth on histopine medium

* exoct means that the transconjugant was selected on octopine selective medium

≁ mutant of exoct1 selected for growth on arginine medium

≠ exarg means that the transconjugant was selected on arginine selective medium

ability to catabolise arginine has been transferred from the donor to the recipient and that in the majority of cases the Ti plasmid was not transferred to these transconjugants. These transconjugants that have inherited this non-Ti plasmid-linked ability to grow on arginine as carbon source is designated ARGCAT (for arginine catabolism) transconjugants.

Per donor transfer frequences of pTi-1001 and ARGCAT

Experiments were carried out to quantify the transfer frequency of pTi-1001 and of the appearance of ARGCAT transconjugants in a cross between strain 1001 to C58CIRifStr. On day 1, strain 1001 was plated on octopine medium and on arginine medium. On the same day, the recipient strain C58CIRifStr was plated on octopine selective medium and on arginine selective medium. On day 2, the cells of strain 1001 were suspended in sterile water from the octopine and arginine plates to give a cell density of approximately 10^8-10^9 cells/ml and transferred to separate tubes. The cells from the octopine medium were diluted by 10 to give dilutions of 10^0 to 10^{-7}. Ten µl aliquots of the dilutions 10^{-6} to 10^{-4} were spotted in triplicate onto the surface of minimal agar plates to determine the viable count. Ten µl aliquots of the dilutions of 1001 from 10^{-6} to 10^0 were also spotted in triplicate onto the lawn of C58CIRifStr growing on the octopine and arginine selective plates. The 10^0 dilution of the donor precultured on arginine was also spotted onto the recipient lawn on octopine and arginine selective medium to give an indication of whether any transfer occurred without induction by octopine. As a further control, 10 µl aliquots of the 10^0 donor dilutions pregrown on octopine and arginine

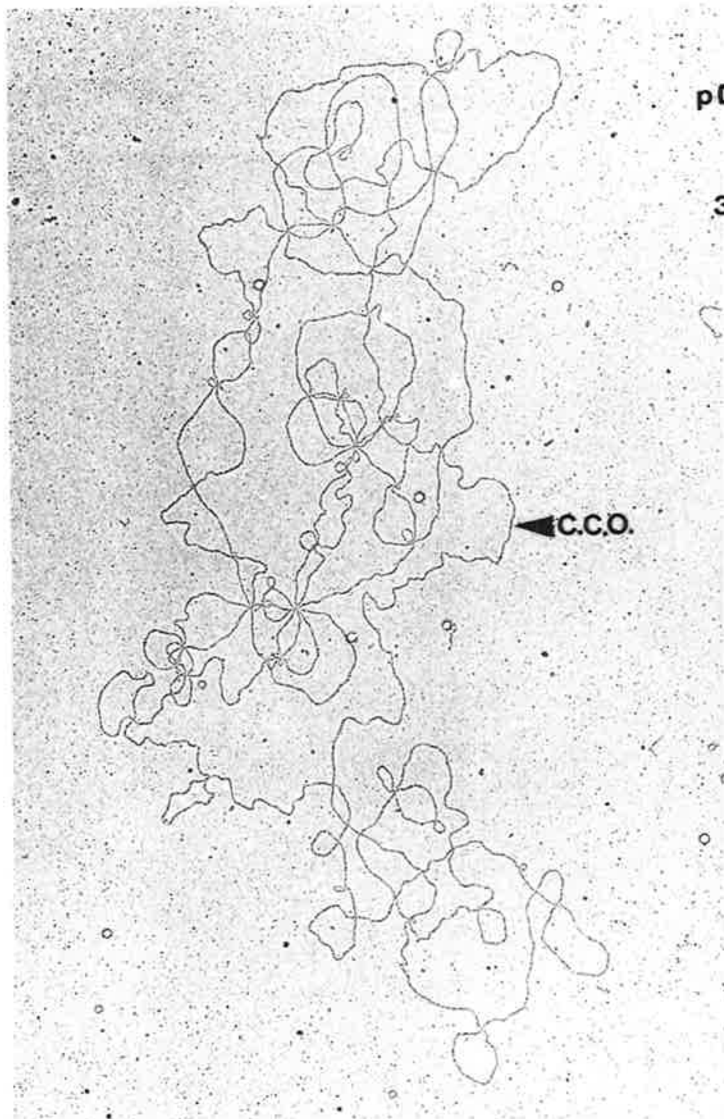
medium were spotted onto both selective media without the recipient. This gave an indication of whether any mutation of the donor to antibiotic resistance had occurred. Octopine-using transconjugants appeared on the selective plates after 2 to 3 days and arginine-using transconjugants appeared more slowly, after about 10 days. No colonies arose in the controls indicating that preculture on octopine was necessary for both Ti plasmid transfer and for the appearance of ARGCAT transconjugants. The frequency of transconjugants per donor was calculated. The frequency of ARGCAT transfer per Ti plasmid was also calculated. The transfer frequency of the Ti plasmid was 2.3×10^{-2} and the transfer frequency of ARGCAT was 4×10^{-5}. This means that there were 1.7×10^{-3} ARGCAT transfers per Ti plasmid transfer.

With the aid of a dissecting microscope and sterile needle, 17 independent ARGCAT colonies were carefully picked from the selective plate and suspended in a drop of sterile water. These suspensions were then streaked on arginine and octopine. All the colonies grew on the arginine medium, but only four grew on octopine medium. Therefore, the majority of these ARGCAT isolates did not inherit the Ti plasmid.

Is ARGCAT due to a plasmid?

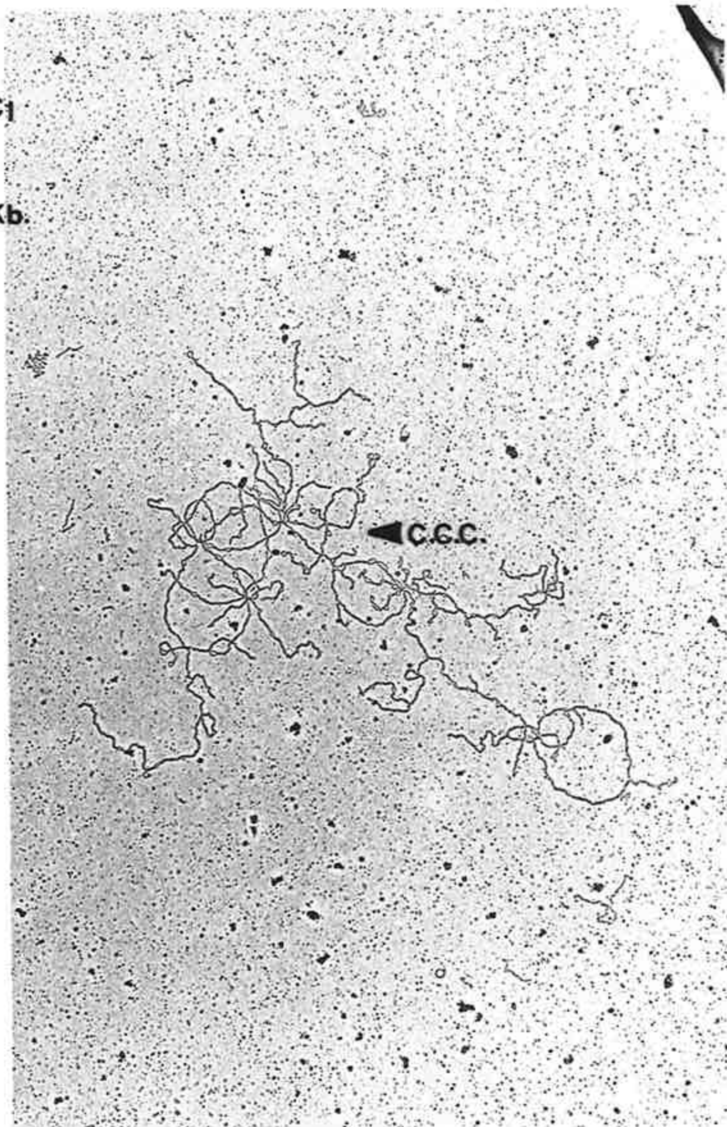
One hypothesis to account for the transfer of arginine catabolism is that the octopine Ti plasmid mobilizes another plasmid which is involved in arginine catabolism and resistance to phage S5. Experiments were carried out to detect this plasmid by physical means. At the time these experiments were begun, strain C58CI, the recipient in the

Figure 4-2. Electronmicrograph of open circular (CCO) and supercoiled (CCC) molecules of pAt-C58 (Casse *et al.*, 1979) isolated from strain C58C1.



pC58C1

386 Kb.



experiments described above, was considered plasmidless. Therefore, an ARGCAT transconjugant exarg1 (Table 4-1) that did not have any Ti plasmid markers was chosen for the detection of a possible ARGCAT plasmid. A single large plasmid was detected in this strain by agarose gel electrophoresis. This plasmid was isolated by caesium chloride-ethidium bromide density gradient centrifugation method when I visited Professor Schell's laboratory in Ghent, and an open circular molecule (Figure 4-2) was measured to have a contour length of 10μm (R. Villarroel, personal communication). Subsequently, it became generally known that C58CI contains a large phenotypically cryptic plasmid of this size (pAt-C58, Casse *et al.*, 1979). The plasmid in the transconjugant exarg1 was indistinguishable from pAt-C58 in the recipient by electrophoretic mobility. A more extensive examination by agarose gel electrophoresis was made of the plasmid content of the donor strain 1001, the recipient C58CIRifStr and several transconjugants (Figure 4-3a). Two plasmids were definitely identified in strain 1001. The smaller was identified as the Ti plasmid by comparison with the plasmid profile of the transconjugant strains which had acquired virulence. The larger plasmid is phenotypically cryptic; it is not in any of the ARGCAT transconjugants, therefore cannot be implicated as an ARGCAT plasmid. Strains such as exarg1 cannot be distinguished from the recipient on the basis of plasmid content.

In some preparations of DNA from 1001, a faint band could be detected in gels that had the same mobility as pAt-C58. If this were the ARGCAT plasmid, then it would not be detected in the ARGCAT transconjugants because it would have the same mobility as pAt-C58.

It is difficult to decide whether this band is a third plasmid in strain 1001 or whether it is due to open circular forms of the two plasmids harboured in strain 1001. The reason for this is the faintness of this band. The plasmid pAt-C58 which has the same electrophoretic mobility as this band is consistently recovered in much larger quantities by the method used in these experiments. Therefore, unless the third band in strain 1001 preparations is due to a plasmid that is inherently less stable than pAt-C58 in the isolation procedure, the hypothesis that this band is due to open circular DNA seems more probable.

Further evidence was sought for this interpretation. If the third band in strain 1001 was the ARGCAT plasmid, its identification in an ARGCAT transconjugant would require a recipient strain that did not contain pAt-C58 and could not use arginine as carbon and nitrogen source. Strain K264CIRifStr is such a strain. This strain is derived from strain C58 (K230 in A. Kerr's collection) received from R. Hamilton, Pennsylvania in 1974. It cannot be distinguished from C58 received from J. Schell, Ghent, using a range of typing bacteriophages. However, K230 contains a cryptic plasmid much smaller than pAt-C58 (Figure 4-4). Strain K264CI was obtained by curing K230 of its Ti plasmid by growth at 37°.

The phenotype of ARGCAT transconjugants derived from donor strain R10 and recipient strain K264CIRifStr are shown in Figure 4-3b. (Strains R10 and 1001 were both isolated in Rumania from grapevines and cannot be distinguished by a range of typing bacteriophage (Chapter 2) and are probably identical.) Figure 4-3b shows the plasmid profiles of strain R10, 264CIRifStr and several transconjugants. Correlation between

Figure 4-3a. Agarose gel electrophoresis of plasmid DNA from 1001, C58ClRifStr and independent transconjugants from the cross 1001 x C58ClRifStr selected on arginine selective medium.

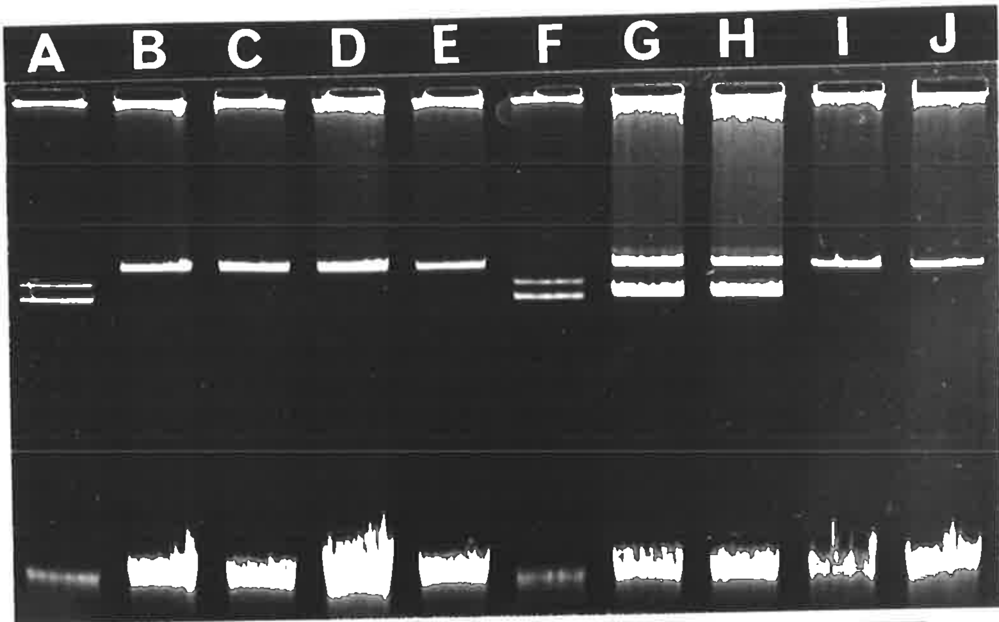
A=F	=	1001
B	=	exarg1
C	=	exarg2
D	=	exarg3
E=J	=	C58ClRifStr
G	=	exarg4
H	=	exarg13
I	=	exarg6

The transconjugant exarg6 is avirulent, occ⁻ and resistant to phage S5. The phenotypes of the other strains are given in Table 4-2.

Figure 4-3b. Plasmid profiles of strain R10, 264ClRifStr and independent transconjugants from the cross R10 x 264ClRifStr selected on arginine selective medium.

			Arc	Occ	Virulence	S5 sensitivity
A=E	=	R10	+	+	+	-
B	=	exargA	+	-	-	-
C	=	exargB	+	+	+	-
D	=	exargC	+	+	+	-
F	=	264Cl	-	-	-	+
G	=	exargD	+	-	-	-

(a)



(b)

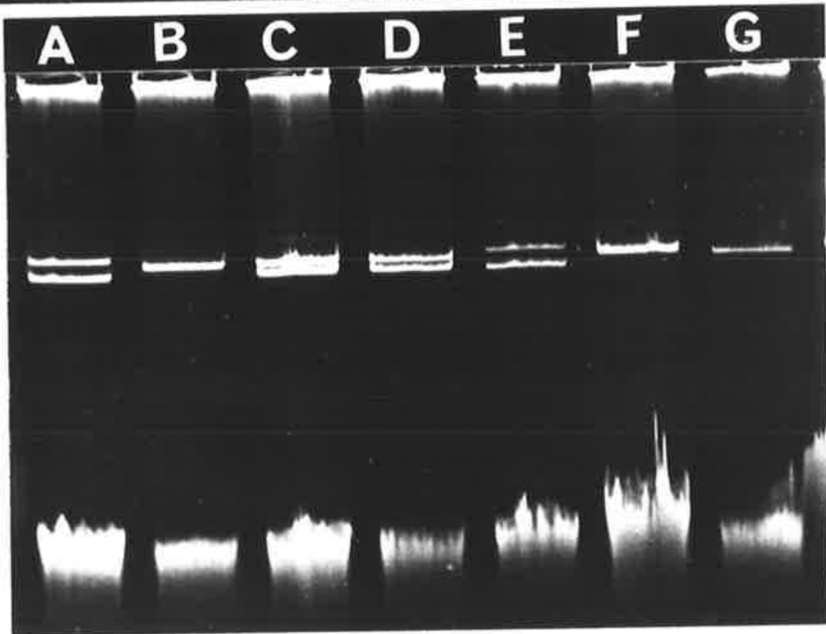
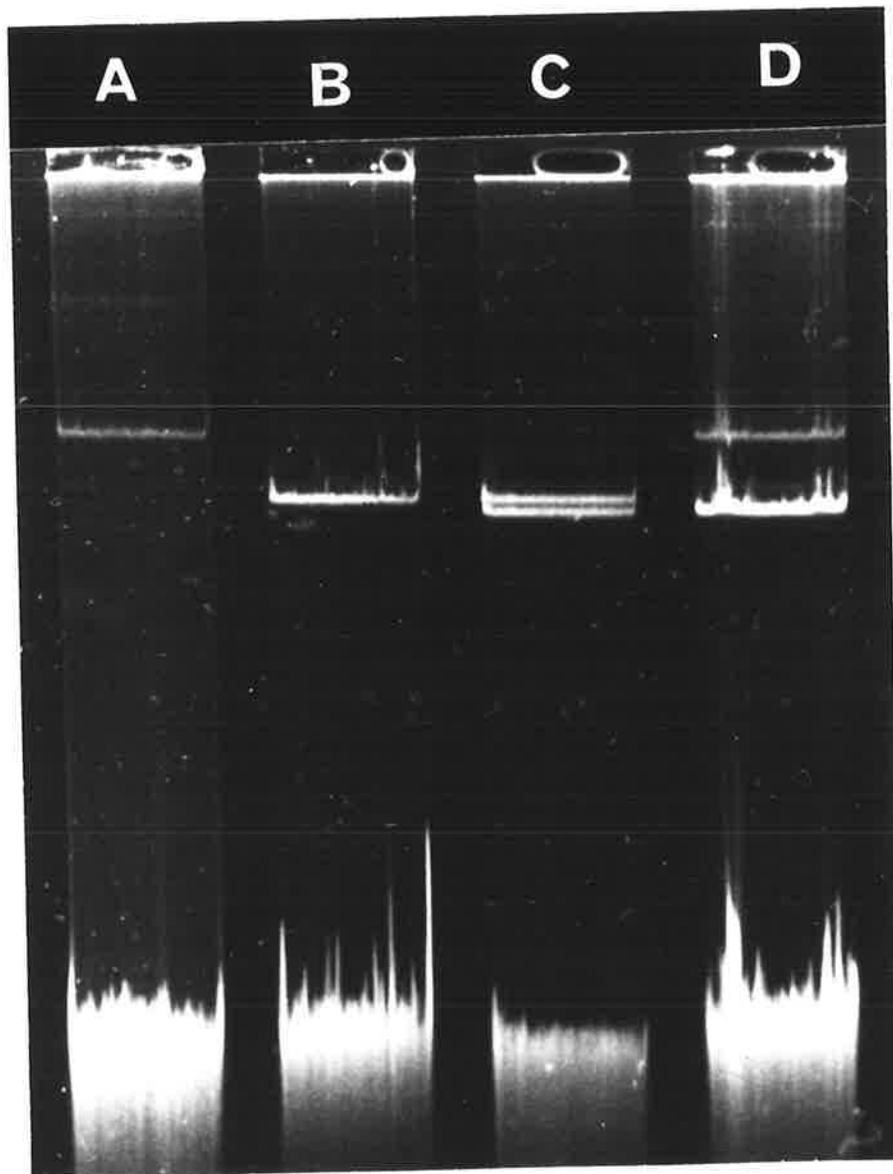


Figure 4-4. Agarose gel electrophoresis of plasmid DNA C58Cl (lane A), C58 (lane D), K230 (lane C) and K264Cl (lane B).


A
B
C
D

phenotype of transconjugants and the presence of a donor plasmid identified the smaller plasmid in R10 as the Ti plasmid. The larger plasmid in R10 has not transferred to any of the transconjugants; therefore there is no evidence that it is involved in arginine catabolism. The cryptic plasmid in 264CIRifStr is present in all the transconjugants. Again a faint band of low mobility was evident in the donor and some transconjugants. It was not detected in all ARGCAT transconjugants and is therefore unlikely to be involved in the catabolism of arginine. This faint band is also probably open circular DNA rather than a third plasmid. Further evidence for this interpreation of the data came from plasmid extraction experiments in which this faint band was detected in strain K230 and its cured derivative (data not shown).

DISCUSSION

The important features of the appearance of ARGCAT transconjugants in crosses between strain 1001 and C58CI are as follows. Transfer of ARGCAT was only observed when the donor strain 1001 (or R10) was grown in the presence of octopine. Ti plasmid transfer also occurred only after induction by octopine. Transfer of ARGCAT can occur without the transfer of the Ti plasmid. These transconjugants do not contain the Ti plasmid. In many cases, but not all, ARGCAT transconjugants are resistant to phage S5. The basis of resistance of one of these transconjugants, exargl, was due to the failure of phage S5 to bind to the bacterium (S.M. Hocking, personal communication).

The high frequency of ARGCAT transfer expressed per Ti plasmid transfers and per donor cell suggested that a second plasmid that is mobilized by pTi-1001 may be involved. Although no plasmidless recipient was available to test this hypothesis, two recipient strains

which have cryptic plasmids of different size were used. Plasmid extracts from these strains and their ARGCAT transconjugants were examined by agarose gel electrophoresis. No plasmid that could be implicated in ARGCAT was detected. In some extracts however, faint bands of equal mobility in the donor strains and ARGCAT transconjugants were detected. Reasons have been given for proposing that these bands are due to open circular forms of the other plasmids in these strains, rather than an ARGCAT plasmid. The problem of open circular molecules is one difficulty that must be considered in the interpretation of agarose gel plasmid profiles.

The possibility that an ARGCAT plasmid was present but had the same mobility in agarose as the chromosomal fragments was considered. Such a plasmid would be obscured and therefore would not be seen in electrophoretograms. However, the plasmid content of one of the donors, R10, has been studied by restriction enzyme digestion of plasmid DNA isolated from caesium chloride-ethidium bromide gradients and no evidence for such a small plasmid has been found (M.D. Chilton, personal communication). Therefore, there is no evidence that the ARGCAT phenotype is controlled by a plasmid.

So, what is the explanation for ARGCAT transfer? Chromosomal gene transfer by the Ti plasmid must be considered. The data on resistance to phage S5 support this case. Most ARGCAT transconjugants are resistant to this phage but some are sensitive. If the genes for S5 resistance and arginine catabolism were closely linked on the chromosome, the S5 sensitive ARGCAT transconjugants may have arisen

after recombination between these closely linked markers. If chromosomal gene transfer has taken place, the mechanism must be interesting to account for the frequent transfer of ARGCAT without the Ti plasmid. A similar situation occurs when the R-factor R68-45 is used to mobilize chromosomal markers in *Pseudomonas aeraginosa*. In most recombinants, R68-45 becomes unstable and is lost during their purification (Haas and Holloway, 1978). Beringer and Hopwood (1976) suggested that the high frequency chromosomal gene transfer by R68-45 may be due to incorporation of DNA sequences into the plasmid that allow it to interact with the chromosome. Regions of homology between plasmid and chromosome may be important for chromosome mobilization. The gene(s) for arginine catabolism on the Ti plasmid (Chapter 3) and on the chromosome (or wherever they are in strain 1001) may supply such regions of homology. Further genetic analysis is obviously necessary to understand ARGCAT transfer.

CHAPTER 5

BIOSYNTHESIS OF AGROCINS AND THE BIOLOGICAL CONTROL OF CROWN GALL

INTRODUCTION

A method of biological control of crown gall developed in South Australia is being used successfully in many parts of the world. Susceptible planting material is dipped into a suspension of living cells of the avirulent strain 84 of *Agrobacterium radiobacter* var. *radiobacter*. The application and mechanism of control has been reviewed (Moore and Warren, 1979; Kerr, 1980).

Kerr and Htay (1974) reported that biological control was due to production by strain 84 of a diffusible antibiotic which these authors referred to as a bacteriocin because of its high degree of specificity for certain strains of *A. radiobacter*. The bacteriocin is called agrocin 84 and its structure (see Figure 5-3) has been elucidated by Tate *et al*. (1979). The evidence presented by Kerr and Htay (1974) that agrocin 84 was the active factor in biological control was the extremely high correlation they found between sensitivity of pathogenic strains to agrocin 84 in an agar plate bioassay and the susceptibility of these strains to biological control in glasshouse tests. Only strains sensitive to agrocin 84 were subject to biological control. In this chapter, further evidence is presented that agrocin 84 is involved in biological control and a preliminary genetic analysis of the biosynthesis of agrocin 84 is described. Several other agrocin producing strains were also studied.

EXPERIMENTAL AND RESULTS

Section A. Biosynthesis of Agrocin 84 is Associated with a Plasmid

Experiments were undertaken to identify the genetic basis of agrocin 84 biosynthesis. The conjugative properties of one of the plasmids resident in strain 84 were used in an effort to mobilise the ability to produce agrocin 84. This plasmid, called pAt-84b (Sciaky *et al.*, 1977) has been measured as 124×10^6 daltons (Merlo and Nester, 1977). It encodes nopaline catabolism enabling its host to grow on nopaline as sole source of carbon and nitrogen. (Such non-oncogenic nopaline catabolic plasmids are referred to as NOC plasmids in this thesis.) This plasmid can be transferred from strain 84 after induction of transfer functions by either nopaline or agrocinopine A (Chapter 7). When strain 84 is plated on octopine as sole source of nitrogen or of carbon and nitrogen, mutant colonies arise that grow faster than the parent. These are regulatory mutants of the nopaline catabolic system (Petit and Tempé, 1975; Petit and Tempé, 1978) and are referred to as Noc^c mutants. The mutant strain $84Noc^c$ used in the present study is also constitutive for transfer of pAt-84b; that is, it does not require induction by opines for conjugation (Ellis and Kerr, 1979).

The plasmid $pAt\text{-}84bNoc^cTra^c$ was transferred by conjugation from strain 84 to recipient strains C58ClRifStr, B9105RifStr and K57RifStr. The donor, strain $84Noc^cTra^c$, was applied as patches on a master plate of conjugation medium (0.2 mg/ml octopine as sole source of carbon and nitrogen). Approximately 5×10^8 recipient cells resistant to rifampicin and streptomycin were spread on a separate plate of conjugation medium.

Both plates were incubated overnight at 27°C and then the donor was replica plated onto the recipient lawn. After 24h this plate was replica plated onto selective medium. Selection was for growth on octopine as the carbon and nitrogen source and for resistance to rifampicin (25 g/ml) and streptomycin (500 g/ml). The transconjugants were purified on the same medium and single colony isolates were tested for phagetype and production of agrocin 84 using the replica plating technique of Kerr and Panagopoulos (1977). All the putative transconjugants had the recipient phagetype. In some experiments, as many as 17% of the Noc^{c} transconjugants were bacteriocinogenic. Examination of the plasmid profiles of donor, recipient and transconjugants from the cross 84Noc^{c}Tra^{c} x C58RifStr (Figure 5-1) shows that strain 84 contains three plasmids (lane E). Correlation of phenotypes and plasmid profiles enables the identification of plasmids. The plasmid of intermediate size in strain 84 Noc^{c}Tra^{c} is pAt-84bNoc^{c}Tra^{c}. The smallest plasmid transferred to the transconjugant in lane F is associated with the agrocin 84 biosynthesis and is referred to as pAt-84a. The largest plasmid in strain 84, designated pAt-84c, is a cryptic plasmid, that is, it has no identified genetic marker. It is slightly smaller than the cryptic plasmid of C58CI (pAt-C58, Casse *et al.*, 1979) as judged by its electrophoretic mobility.

Schell and Van Montagu (personal communication) have also identified pAt-84a as the bacteriocinogenic plasmid in strain 84 by mobilization with pAt-84b. They have also mobilised an agrocin plasmid from NCPPB396 using the R factor, RP4.

Although agrocin 84 biosynthesis is associated with pAt-84a,

Figure 5-1. Agarose gel electrophoresis of plasmid DNA.

A (590 Noc^c x 398RifStr#5) x C58ClChl; avirulent, non-bacteriocinogenic.

B (590 Noc^c x 398RifStr#5) x C58ClChl; avirulent, bacteriocinogenic

C 398RifStr; bacteriocinogenic.

The strains shown in lanes A, B and C are described in Section B and in Figure 5-5.

D C58Cl(RP4) (= GV3805 from J. Schell).

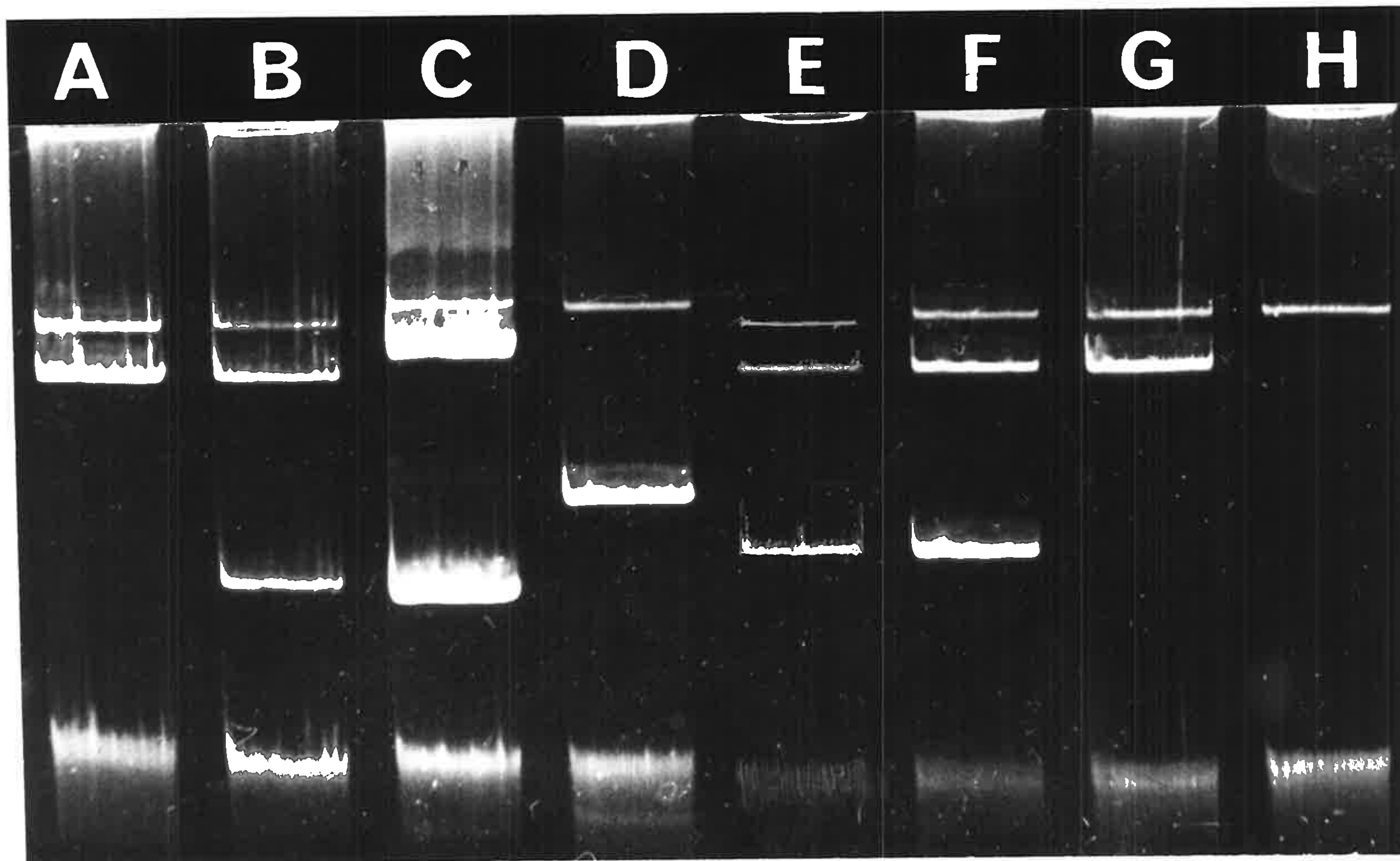
E Strain 84 derivative, 84 Noc^c, bacteriogenic.

F C58ClRifStr (pAt-84b Noc^cTra^c)(pAt-84a) bacteriogenic.

G C58ClRifStr (pAt-84b Noc^cTra^c) non-bacteriocinogenic.

H C58ClRifStr (= GV4 from J. Schell).

The agrocin plasmids are the plasmid bands of highest mobility in lanes B, C, E and F. Their mobility can be compared with that of RP4 (mol. wt 34 x 10^6 daltons), the band of highest mobility in track D.


A
B
C
D
E
F
G
H

the data presented above do not eliminate the possibility that pAt-84a and pAt-84b are both involved since no transconjugant was isolated that received only pAt-84a. The isolation of this type of transconjugant is described below.

A transconjugant strain, C58CIRifStr (pAt-84b)(pAt-84a) was constructed as described above using nopaline to induce transfer of the NOC plasmid pAt-84b from wild type strain 84. The transconjugant was selected for its ability to grow on nopaline. This strain was inducible for nopaline catabolism and consequently was unable to grow on octopine medium. It was used as recipient in a second conjugation with donor strain C58CI(pWI1009). This strain harbours a Tra^{c} derivative of pTi-C58 which also confers the ability to grow on octopine medium (see Chapter 8). Transconjugants were selected for the donor's ability to grow on octopine and the recipient markers rifampicin and streptomycin resistance. These transconjugants were virulent. The plasmid profiles of the donor, C58CI(pWI1009) (lane A), the recipient C58CIRifStr (pAt-84b)(pAb-84a) (lane B) and a virulent transconjugant C58CIRifStr (pWI1009)(pAt-84a) (lane C) are shown in Figure 5-2. The Ti plasmid in the donor (lane A) is larger (lower relative mobility) than the NOC plasmid pAt-84b (lane B). The transconjugant (lane C) has a plasmid of the same relative mobility as the Ti plasmid. The unselected NOC plasmid pAt-84b has been lost, presumably because it is incompatible with the Ti plasmid. Although the transconjugant (lane C) has the bacteriocinogenic pAt-84a, it does not produce agrocin 84. The reasons for this are discussed later in Chapter 8.

The Ti plasmids in strain C58 and its derivative are temperature sensitive for replication and can be eliminated by growth at 37°C

Figure 5-2. Agarose gel electrophoresis of plasmid DNA.

A = C58C1(pWI1009), a Noc^c mutant of GV3804 from J. Schell.

pWI1009 is a Tra^c derivative of pTi-C58 (see Chapter 8).

B = C58ClRifStr (pAt-84b)(pAt-84a).

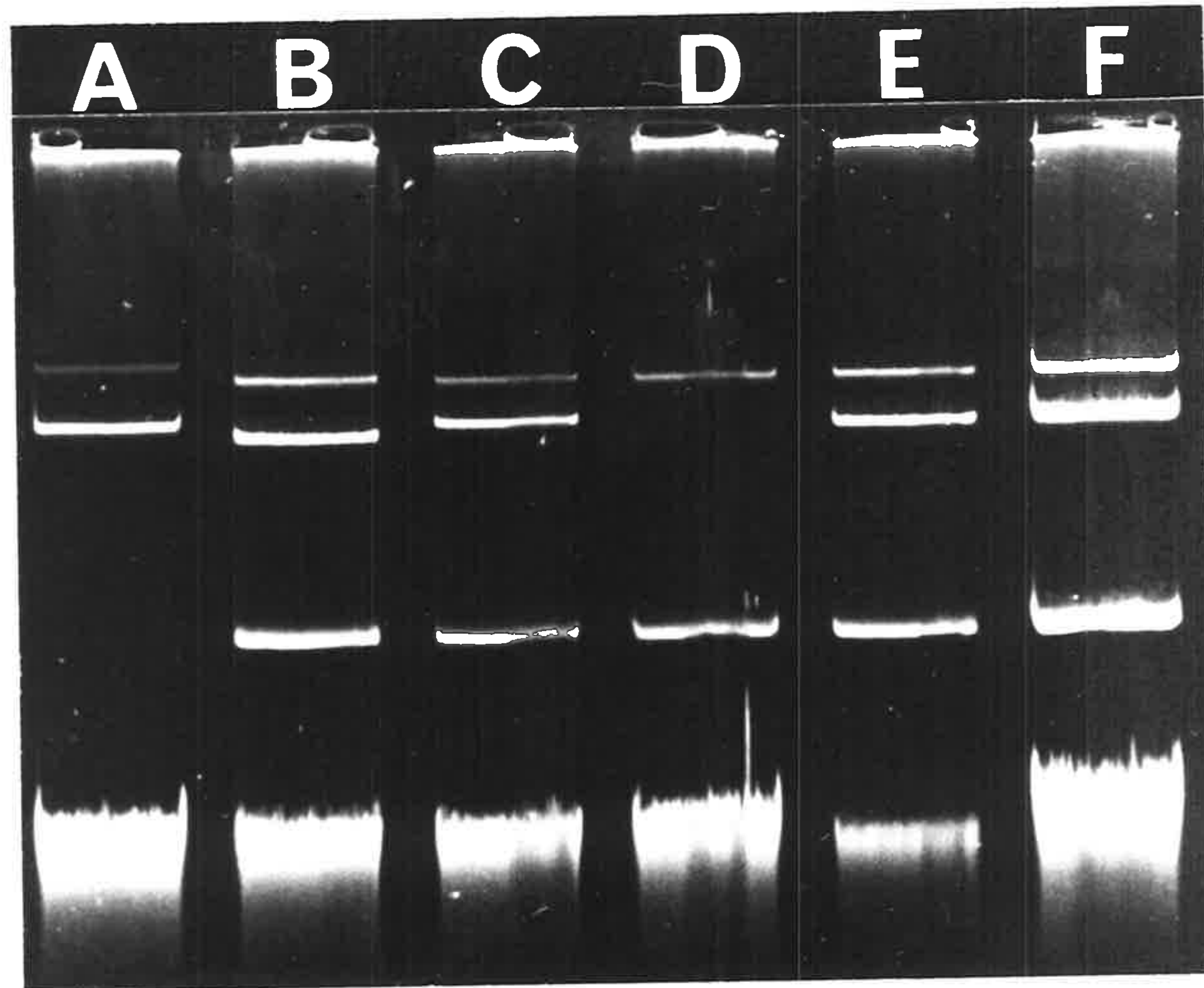
C = C58ClRifStr (pWI1009)(pAt-84a).

D = C58ClRifStr (pAt-84a).

E = C58ClRifStr (pTi-C58)(pAt-84a).

F = C58ClRifStr (pWI1011)(pAt-84a).

The plasmid pWI1011 is an Agr^r mutant derived from pWI1009 and is described in Figure 8-5.


A
B
C
D
E
F

(Hamilton and Fall, 1971; Van Larebeke *et al.*, 1974). The transconjugant in lane C was grown at 37° and then plated for single colonies on octopine medium. Many small colonies appeared that were unable to use octopine. One colony was purified for further examination. Its plasmid content is shown in Figure 5-2, lane D. It has lost the Ti plasmid and is consequently avirulent and produces agrocin 84. This strain is called C58CIRifStr(pAt-84a). Because it produces agrocin 84, it is obvious that the NOC plasmid pAt-84b has no role in agrocin 84 biosynthesis.

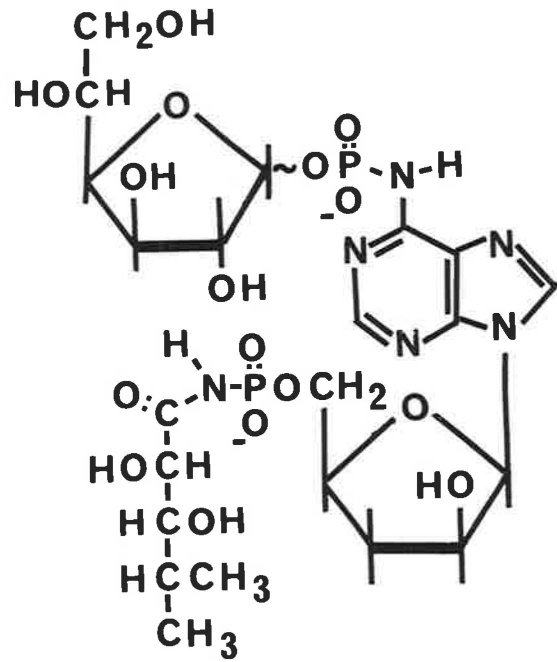
The bacteriocinogenic pAt-84a confers immunity to FNT

Tate *et al.* (1979) have reported a second antibiotic substance, possibly a degradation product of agrocin 84, that is produced by cultures of strain 84. The structure of this compound which is referred to as FNT (fast nucleotide) and that of agrocin 84 are shown in Figure 5-3. FNT has a broad antibiotic spectrum against many strains of *A. radiobacter* and unlike agrocin 84, sensitivity to FNT is not determined by the Ti plasmid. From a structural analysis of the two compounds, Tate *et al.* (1979) have shown that the bacteriocin-like specificity of agrocin 84 is conferred by a D-glucofuranosyloxyphosphoramidate linked to the 6 position of adenine (Figure 5-3). This moiety is involved in the recognition and uptake of agrocin 84 by sensitive strains of *Agrobacterium* (P.J. Murphy, personal communication) and can be lost by thermal degradation of agrocin 84 to produce FNT (Tate *et al.*, 1979).

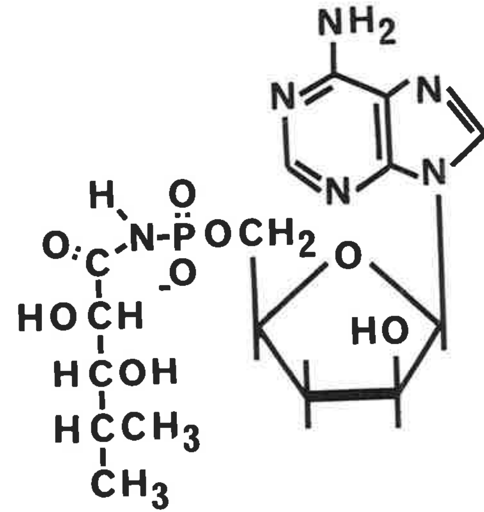
Strains C58CIRifStr and C58CIRifStr(pAt-84a) were tested for sensitivity to FNT. Twenty µl of a solution of FNT supplied by

Figure 5-3. Structures of agrocin 84 (A) and its thermal degradation product referred to in this thesis as FNT (B).

A



B



Dr M.E. Tate at a concentration of approximately 0.48mM was added to a well cut into the centre of plates of Stonier's agar. After 3h, when the solution had diffused into the agar, the plate was sterilized with chloroform vapour and overlayed with the indicator strains in soft agar and incubated at 27° for 36h. Strain C58Cl was sensitive; a clear zone of inhibition with a diameter of 2 cm resulted. No resistant mutants appeared within the zone of inhibition. Strain C58CIRifStr(pAt-84a) was insensitive. Resistance to FNT therefore appears to be a selectable marker for the bacteriocinogenic plasmid pAt-84a. It should therefore be possible to manipulate this plasmid in genetic crosses as an R-factor using FNT as the selective antibiotic. Using this method, further genetic analysis of this plasmid should be facilitated.

Transfer of pAt-84a to virulent agrobacteria

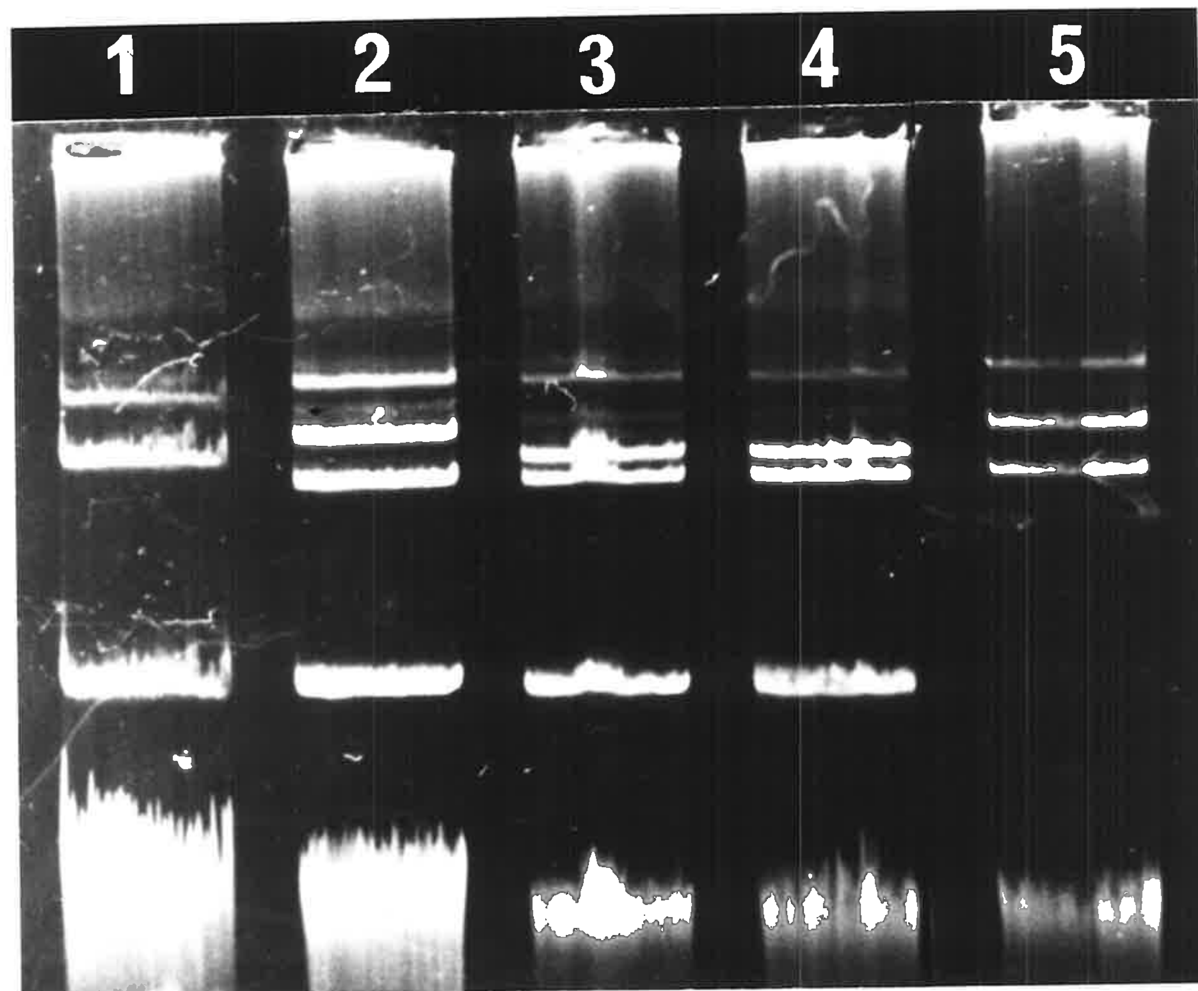
Panagopoulos *et al.* (1979) reported transfer of the ability to produce agrocin 84 to pathogenic strains of *A. radiobacter*. Transfer occurred during biological control experiments. Although the virulent recipient strains were sensitive, the agrocin 84-producing pathogens were insensitive to agrocin 84 and were not subject to biological control. The genetic basis of these observations was investigated and has been reported (Ellis and Kerr, 1979). In these experiments, strain 84Noc^{c}Tra^{c} was crossed with a pathogenic recipient strain K57RifStr(pTi-27) as described previously for the cross between 84 Noc^{c}Tra^{c} and C58CIRifStr. The recipient harbours a nopaline Ti plasmid, is sensitive to agrocin 84 and cannot grow on octopine medium. The same replica plating procedure described earlier was used to select for octopine-using transconjugants.

Figure 5-4. Transfer of pAt-84a to a virulent strain of *A. radiobacter*. Agarose gel electrophoresis of plasmid DNA.

1. C58ClRifStr (pAt-84b $Noc^{c}Tra^{c}$)(pAt-84a).
2. Virulent transconjugant from the cross 84 $Noc^{c}Tra^{c}$ x K57RifStr(pTi-27).

3 & 4. Avirulent Noc^{c} transconjugants from this cross. pTi27 has been displaced by pAt-84b $Noc^{c}Tra^{c}$.

5. K57RifStr(pTi-27), the virulent recipient strain. The band of intermediate mobility is pTi-27 (unpublished data).


1
2
3
4
5

The patches of transconjugants on selective plates were touched with a loop and streaked for single colonies on octopine medium. Single colonies that used octopine and single colonies that did not* were tested for agrocin 84 production. In one experiment, 9 colonies that grew on octopine were tested for agrocin 84 production and of these, 5 were positive. These agrocin 84 producers were avirulent. Of 27 single colonies that did grow on octopine, 10 produced agrocin 84. These were virulent. Examination of the plasmid profiles of some of these strains showed that the agrocin 84 producers contained pAt-84a (Figure 5-4). The virulent agrocin 84-producing strain (lane 1) contains pTi-27 which is the plasmid of intermediate size in the recipient (lane 5). The transconjugants on lanes 3 and 4 have lost pTi-27 and harbour pAt-84b $Noc^{C}Tra^{C}$. Presumably pTi-27 and the NOC plasmid pAt-84b$Noc^{C}Tra^{C}$ are incompatible.

Results presented by Ellis and Kerr (1979) indicate bacteriocinogenic plasmid pAt-84a is mobilised by pAt-84b$Noc^{C}Tra^{C}$. It is not known whether pAt-84a is a self-transmissible plasmid that can transfer without pAt-84b. Experiments using C58CIRifStr(pAt-84a) as donor and FNT to select transfer of pAt-84a may answer this question.

Section B. Crosses Involving NCPPB398

Strain 398 is a biovar 1 strain; it is virulent and induces galls that contain neither octopine nor nopaline. Neither can it catabolise these opines (Kerr and Roberts, 1976). Since the work described in this section was done, it has been shown that strain 398 can

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* These colonies were minute and grew on impurities in the agar.

grow on 'mannopine' which is a mannose-glutamic acid condensation product related to agropine (Tempé *et al.*, 1980); it can catabolise agrocinopine C (Chapter 6) and can be induced to transfer its Ti plasmid by agrocinopine C (Chapter 7). These are the properties of agropine strains such as Bo542. So, although the tumours induced by this strain have not yet been examined for agropine, strain 398 is most likely an agropine strain.

Strain 398 produces an agrocin of the same specificity as agrocin 84. These agrocins are probably identical (Roberts, personal communication). The genetic basis of agrocin 398 production was examined.

Introduction of a conjugative plasmid to strain 398

At the time this work was done, there was no known selectable markers on pTi-398 and the control of conjugation of this plasmid had not been elucidated. Therefore, in an effort to mobilise the genes for synthesis of agrocin 398 as had been done for strain K84, a conjugative plasmid with a selectable trait was introduced into strain 398. A nopaline catabolic (NOC) plasmid from strain 590 was used. This strain grew on nopaline, was sensitive to agrocin 84 and was avirulent. A mutant of this strain was selected that could grow on octopine and was designated strain 590Noc^{c}. Strain 590Noc^{c} was crossed with strain 398RifStr with selection for the donor Noc^{c} phenotype (growth on octopine) and the recipient's resistance to 25μg/ml rifampicin and 500μg/ml streptomycin. The recipient was spread on a plate of minimal agar containing 0.2mg/ml octopine and the donor was applied as 10 patches

to a second Petri dish of the same medium. After one day's growth, the donor patches were replica plated on to the recipient lawn. After a further day, the conjugation plate was replica plated to a plate of selective medium containing 0.2mg/ml octopien as nitrogen source and 0.5% glucose plus rifampicin (25µg/ml) and streptomycin (500µg/ml). Transconjugants appeared after 3-4 days and were purified through a single colony on the selective medium. Ten independent transconjugants were purified (one from each patch on the selective plate). They were tested for virulence on tomato seedlings, octopine utilization (by streaking on 2mg/ml octopine) and for agrocin production. Nine transconjugants were virulent and one was avirulent. The reason for loss of virulence will be discussed later. All utilized octopine, produced agrocin 398 and had the typical rough colony morphology of strain 398 on YM agar. The one avirulent transconjugant referred to as 590Noc^{C} x 398RifStr#1 and a virulent transconjugant 590Noc^{C} x 398RifStr#5 were tested and gave the phagetype of strain 398.

Transfer of agrocin 398 biosynthesis to C58CI

Having introduced a selectable conjugative plasmid into strain 398RifStr, one transconjugant was used as a donor in a second round of conjugation. The virulent transconjugant 590Noc^{C} x 398RifStr#5 was used as donor and C58CIChl as recipient. Transconjugants were selected on octopine as sole source of carbon and nitrogen and chloromycetin (125µg/ml) to counter-select the donor. Seventy-five transconjugants were purified and tested for virulence. Sixteen were virulent. Eleven transconjugants were tested for agrocin 398 biosynthesis and seven were positive. The seven producers were immune to agrocin 84 and the

Table 5-1. Characteristics of the strains involved in crosses with strain 398RifStr or its derivatives.

	Octopine utilisation	Agrocin 84 production	Agrocin 84 ≠ sensitivity	Virulence
$590Noc^{C}$	+	-	+	-
398RifStr	-	+	-	+
$590Noc^{C}$x398RifStr#1	+	+	-	-
#5	+	+	-	+
C58-ClChl	-	-	-	-
($590Noc^{C}$x398RifStr#5) xC58-ClChl				
Transconjugant class A	+	+	-	+
B	+	-	+	-
C	-	-	+	+
D	+	+	-	-

≠ + = sensitive; - = not sensitive.

remaining non-producers were sensitive. Several interesting points emerged from these crosses. First, the ability to use octopine could be transferred to an agropine strain and in one out of ten transconjugants, virulence was lost. (No spontaneous loss of virulence has been observed in the recipient strain 398.) Secondly, the ability to produce agrocin 398 was transferred to C58CICh1 and in the following section it is shown that agrocin 398 synthesis is controlled by a small plasmid referred to as pAt-398a. Thirdly, the agrocin 84 sensitivity marker carried by the NOC plasmid of strain $590Noc^{c}$ is not expressed in a cell that harbours pAt-398a. It is however re-expressed when the NOC plasmid is transferred to a new genetic background without pAt-398a. Therefore pAt-398a encodes immunity to the agrocin it produces.

Plasmid profiles of strain 398 and its derivatives

The strains used as donors and recipients and various transconjugants from the crosses involving strain 398 are listed in Table 5-1. The plasmid profiles of these strains are shown in Figure 5-5. Examination of these profiles can help explain some of the results observed in these crosses. Firstly, why is the transconjugant $590Noc^{c}$ x 398RifStr#1 (lane C) avirulent and $590Noc^{c}$ x 398RifStr#5 (lane D) virulent? Both these transconjugants have a plasmid that is not present in the recipient strain 398 (lanes B and I). In the case of the avirulent transconjugant #1, this extra plasmid corresponds to the smallest plasmid in the donor $590Noc^{c}$ (lane A). This plasmid is probably the NOC plasmid that has been transferred from strain $590Noc^{c}$. The extra plasmid in the virulent transconjugant #5 does not correspond to any plasmids in the donor $590Noc^{c}$ or in the recipient strain 398RifStr. Therefore it may be a cointegrate plasmid involving the NOC plasmid and a plasmid in 398RifStr, probably pTi-398. The

Figure 5-5. Agarose gel electrophoresis of plasmid DNA from strains 590 Noc^{c}, 398RifStr, C58ClChl and transconjugant derivatives. (a) shows the gel photograph and (b) is an interpretation of the plasmid content of these strains. The arrowed features in Figure 5-5b are:

(1) origin.

(2) hazy band of low mobility associated with the bacteriogenic strains.

(3) bacteriocinogenic plasmid derived from 398RifStr.

(4) chromosomal DNA fragments.

A = 590 Noc^{c}, avirulent.

B = 398RifStr, virulent, bacteriocinogenic.

C = 590 Noc^{c} x 398RifStr#1, avirulent, bacteriocinogenic.

D = 590 Noc^{c} x 398RifStr#5, virulent, bacteriocinogenic.

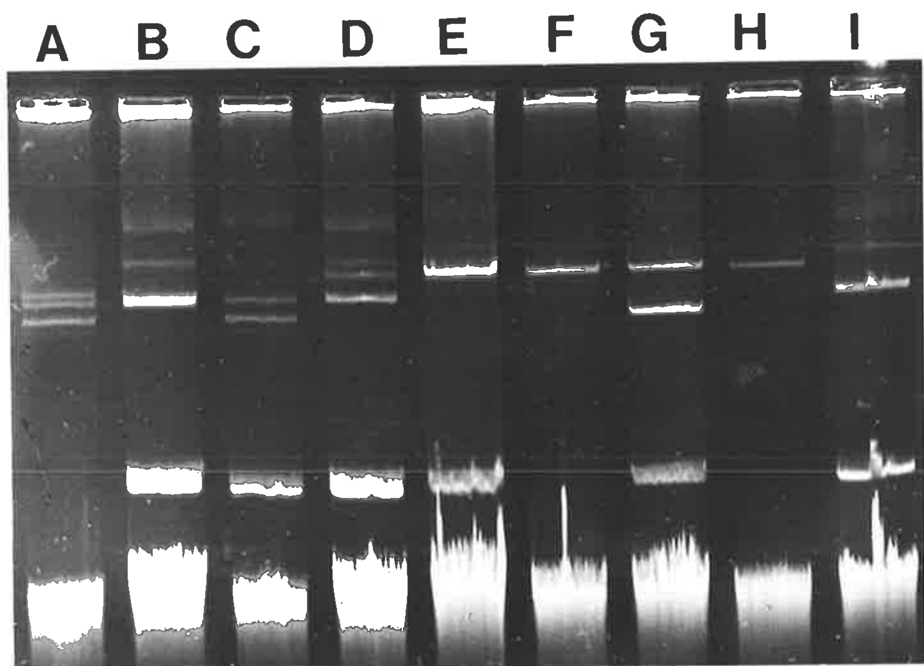
E = (590 Noc^{c} x 398RifStr#5) x C58ClChl, virulent, bacteriocinogenic.

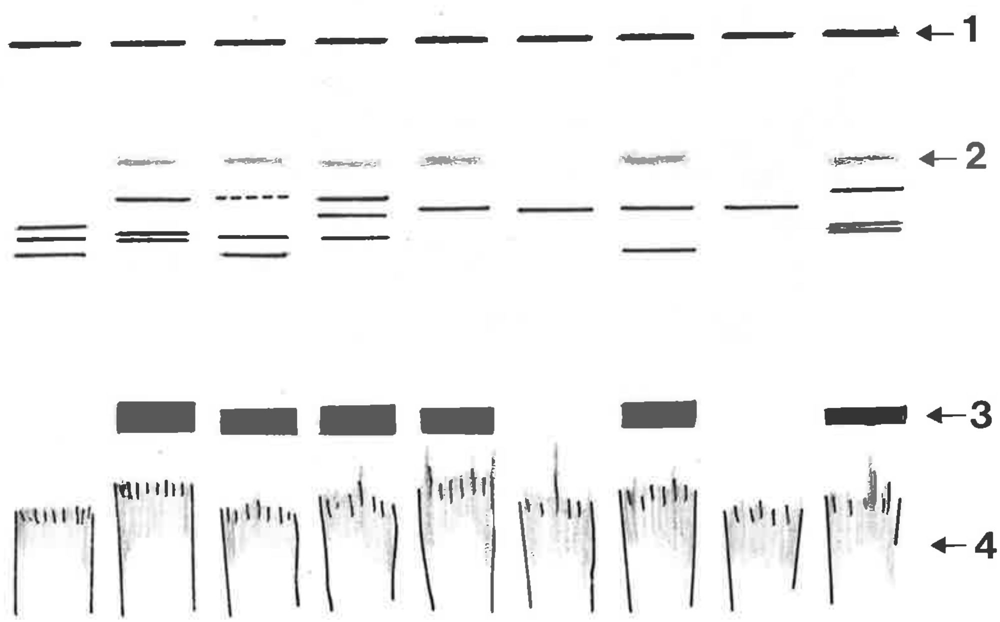
F = (590 Noc^{c} x 398RifStr#5) x C58ClChl, virulent.

G = (590 Noc^{c} x 398RifStr#5) x C58ClChl, avirulent, bacteriocinogenic.

H = C58ClChl.

I = 398RifStr.


A
B
C
D
E
F
G
H
I


←1
←2
←3
←4

fact that a cointegrate has formed implies that the two plasmids are incompatible. In the case of the avirulent transconjugant #1 the NOC plasmid, the selected plasmid, has probably eliminated the Ti plasmid, accounting for the loss of virulence. In the case of the virulent transconjugant #5, a cointegrate has formed that combines the selected character (the Noc^{C} phenotype) and the virulence of pTi-398. This interpretation is supported by the plasmid complement of the transconjugants resulting from the cross (590Noc^{C} x 398RifStr#5) x C58CIChl). Only one large plasmid can be detected in the virulent transconjugants (lanes E and F) and this has the same mobility as pAt-C58 (Casse *et al.*, 1979) in the recipient (lane H). The putative cointegrate in the donor also has the same mobility. Therefore the virulent transconjugants may contain pAt-C58 and the cointegrate. This interpretation is supported by the plasmid profile of the avirulent C58CIChl transconjugant (lane H) which has a plasmid that has the same mobility at pAt-C58. Although the cointegrate is stable (no spontaneous 'disintegrates' are seen in the plasmid profile in the donor) it must break down during conjugation. Formation of the cointegrate is the most common event in the first cross since 9/10 transconjugants were Noc^{C} and virulent. Breakdown of the cointegrate is the most common event in subsequent conjugations since only 16/75 of the transconjugants resulting from the second conjugation are virulent. Formal proof of this scheme will require restriction analysis and homology studies of plasmids in the various strains.

Correlation of agrocin 398 production with plasmid content in the donor strain 398 and the transconjugants in the C58CI background indicates that the smallest plasmid in strain 398 referred to as pAt-398a is the bacteriocinogenic plasmid.

Careful examination of the plasmid profile of strain 398 shows that it contains at least four plasmids, the smallest is pAt-398a, the largest referred to as pAt-398c and two plasmids of very similar mobility that can be resolved as a doublet when the gel is loaded with less DNA and electrophoresed longer (data not shown). One of these plasmids is pTi-398. Also, in many preparations (Figure 5-5 and unpublished data), a hazy band of very low mobility is seen in strain 398 and its derivatives. These bands appear only in those strains harbouring the bacteriocinogenic plasmid of 398 and may be some alternative structure of this plasmid, for example linear molecules arising during plasmid isolation.

Section C. Crosses Involving Bo542

Strain Bo542 is an agropine strain (Guyon *et al.*, 1980) that can be distinguished from strain 398 by its phagetype (Appendix I). It also produces an agrocin of the same specificity as agrocin 84. In Chapter 7, experiments are described in which pTi-Bo542 is transferred by conjugation to the recipient strain C58ClRifStr. Some of the transconjugants that were isolated produced agrocin 542. Evidence is presented in Chapter 8 that agrocin biosynthesis is controlled by a 25×10^6 dalton plasmid in Bo542.

Section D. The Genetic Basis of Agrocin Biosynthesis by Other Bacteriocinogenic Strains

The plasmid content of several other strains which produce agrocins whose specificities differ from agrocin 84 have also been

Figure 5-6. Agarose gel electrophoresis of plasmid DNA from several bacteriocinogenic strains and their derivatives.

A. 108 virulent and bacteriocinogenic.

B. C58ClRifStr(pTi-108) virulent and non-bacteriocinogenic transconjugant.

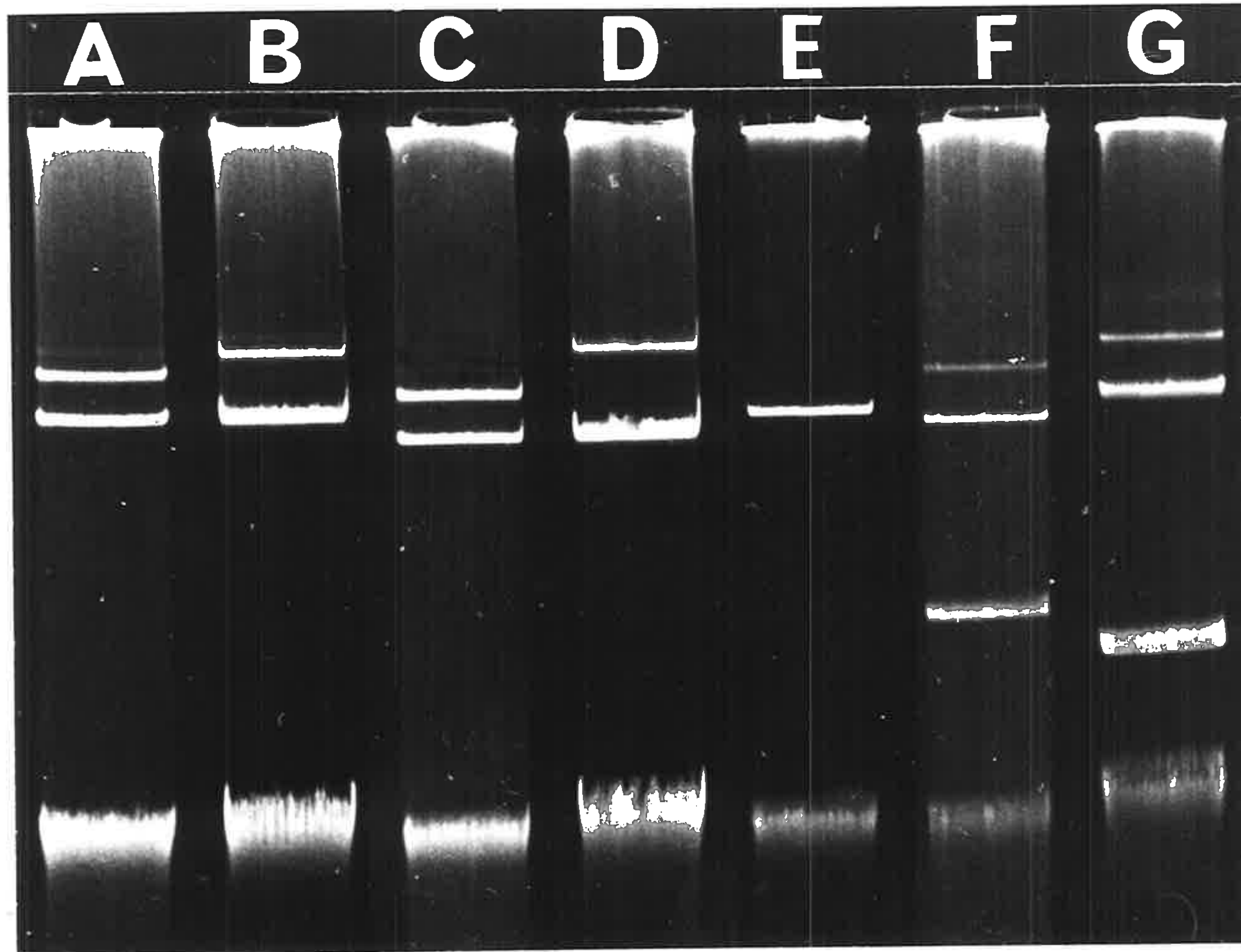
C. 112 avirulent and bacteriocinogenic, harbours a NOC plasmid.

D. C58ClRifStr(pAt-112a) a non-bacteriocinogenic transconjugant. pAt-112a is a NOC plasmid - it encodes nopaline catabolism and agrocin 84 sensitivity.

E. 128 avirulent and bacteriocinogenic.

F. 84 avirulent, bacteriocinogenic.

G. 398 virulent, bacteriocinogenic.


A
B
C
D
E
F
G

investigated by agarose gel electrophoresis. These are strains K108, K112, K128 and Ag40. Strain K108 was of particular interest because its agrocin is a cytidine nucleotide derivative (Elvin and Tate, personal communication). Therefore both agrocin 84 and agrocin 108 are nucleotide bacteriocins. However, no evidence could be found that agrocin 108 biosynthesis is controlled by a plasmid. No plasmids of a similar size to the bacteriocinogenic plasmid of strain 84 were detected in strains K108, K112 or K128 (lanes A, C and E in Figure 5-6) or in strain Ag40 (results not shown). The strain Ag40 which produces an agrocin that inhibits several biovar 2 strains of *A. radiobacter* is interesting because although it is insensitive to agrocin 84 and consequently not subject to biological control, transconjugant strains harbouring pTi-Ag40 are sensitive (Kerr and Roberts, 1976; Ellis, unpublished data). It is possible that the mechanism which protects strain Ag40 from its own agrocin gives cross protection against agrocin 84.

Section E. Biological control by bacteriocinogenic transconjugants

If agrocin 84 is the active factor in biological control of crown gall, the avirulent transconjugants produced above should act as biological control agents. The strains listed in Table 5-2 were tested as control agents in glasshouse tests on tomato stems using the method of Kerr and Htay (1974). Strains K57 (pTi-27) was used as the agrocin 84 sensitive pathogen. Results are presented in Table 5-3 and Figure 5-7. Only those transconjugants that produce agrocin 84 acted as control agents.

Table 5-2. Biological control is associated with production of agrocin 84.

Strains	Agrocin 84 production	Biological control of K57(pTi-27)
84Noc^{c}Tra^c	+	-
C58ClRifStr	-	-
B9105RifStr	-	-
K57RifStr	-	-
C58ClRifStr(pAt-84b Noc^{c}Tra^c)	-	-
C58ClRifStr(pAt-84b Noc^{c}Tra^c)(pAt-84a)	+	+
C58ClRifStr(pAt-84a Noc^{c}Tra^c)	+	+
B910RifStr(pAt-84b Noc^{c}Tra^c)	-	-
B9105RifStr(pAt-84b Noc^{c}Tra^c)(pAt-84a)	+	-
K57RifStr(pAt-84b Noc^{c}Tra^c)	-	-
K57RifStr(pAt-84b Noc^{c}Tra^c)(pAt-84a)	+	+

Figure 5-7. Biological control of pathogenic strain K57(pTi-27) by bacteriocinogenic transconjugants, tested on tomato stems at a ratio of 1:1.

a. A = 57(pTi-27) + sterile water.

B = 57(pTi-27) + C58ClRifStr(pAt-84b $Noc^{c}Tra^{c}$).

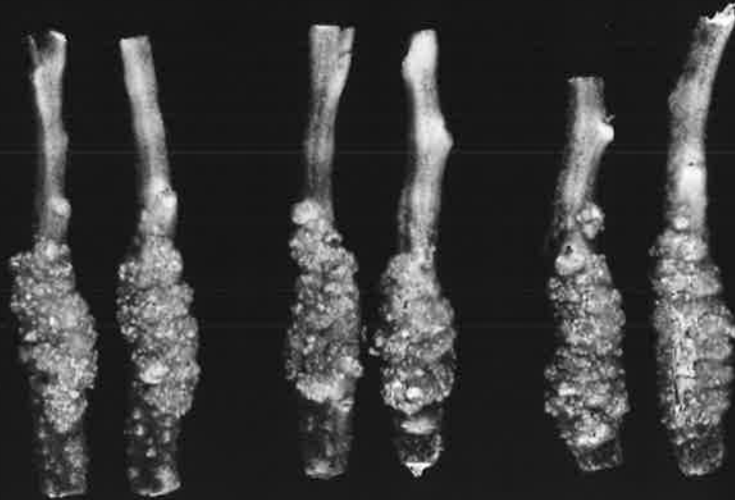
C = 57(pTi-27) + C58ClRifStr(pAt-84b $Noc^{c}Tra^{c}$) (pAt-84a).

b. A = 57(pTi-27) + sterile water.

B = 57(pTi-27) + B9105RifStr(pAt-84b $Noc^{c}Tra^{c}$).

C = 57(pTi-27) + B9105RifStr(pAt-84b $Noc^{c}Tra^{c}$) (pAt-84a).

a
A
B
C


b
A
B
C

The bacteriocinogenic transconjugant in the genetic background of B9105 did not prevent crown gall. Poor growth at the wound site on the tomato stem has been proposed as the reason for this (Ellis *et al.*, 1979b). Also, the transconjugant 590Noc^{C} x 398RifStr#1 did not control crown gall. However, both of these bacteriocinogenic strains were able to control crown gall when a ratio of 10 bacteriocinogenic to one pathogenic cell was used to inoculate the tomato stems.

Section F. The Breakdown of Effectiveness of Biological Control in the Field

Several possibilities for breakdown of the effectiveness of biological control exist. One is the selection of mutants that are resistant to agrocin 84. Sensitivity to agrocin 84 is encoded by the nopaline Ti plasmids (Engler *et al.*, 1975). Resistant mutants can be readily isolated and many of these are avirulent. However, agrocin 84 resistant mutants that are still virulent can be isolated. One such mutant of strain T37 was isolated in this study and was not subject to biological control by strain 84. However, there is no evidence for the selection of such mutants in the field.

A second form of breakdown of biological control was reported by Panagopoulos *et al.* (1979) and is due to the transfer of pAt-84a to pathogenic strains. These strains become immune to agrocin 84 and are not subject to biological control. The genetic basis for this is described earlier in this chapter and means to minimise this danger were proposed by Ellis and Kerr (1979) and will be dealt with in the discussion section in this chapter.

DISCUSSION

In this chapter, evidence has been presented that the biosynthesis of agrocin 84 by the widely used biological control organism, strain 84, is controlled by a small plasmid, pAt-84a (Sciaky *et al.*, 1977). When this plasmid was transferred to certain avirulent strains of *A. radiobacter*, most of the bacteriocinogenic transconjugants were effective biological control agents. This is further evidence in support of the proposal by Kerr and Htay (1974) that biological control occurs through production of agrocin 84.

Two ways in which the effectiveness of biological control may break down have been considered. The first was the selection of agrocin 84 resistant mutants. These mutants can occur by mutation of the Ti plasmid-linked genes involved in agrocin 84 sensitivity. Why don't such mutants arise and spread in the field? These mutants may carry a genetic load. First, agrocin 84-resistant mutants cannot catabolise agrocinopines A and B (Chapter 6), and secondly they may be affected in their ability to transfer their Ti plasmid. Some evidence will be presented in Chapter 6 that agrocin 84 and agrocinopines A and B enter the cell through the same permease. This permease does not operate in agrocin 84 resistant mutants (P.J. Murphy, personal communication). Since agrocinopine A and B are involved in induction of conjugation of nopaline Ti plasmids (Chapter 7), these opines may not be able to enter the agrocin 84-resistant cells. These cells would become effectively transfer deficient which may have some bearing on the maintenance of Ti plasmids carrying the mutation to agrocin 84 resistance.

A second way in which the effectiveness of biological control can break down is through transfer of the bacteriocinogenic plasmid from strain 84 to the pathogen. Such transconjugants are not sensitive to agrocin 84 and are not subject to biological control (Panagopoulos *et al.*, 1979). Evidence has been presented in this chapter that bacteriocinogenic plasmids in *A. radiobacter* confer immunity to their own agrocin. Furthermore, it has been shown that pAt-84a confers immunity to a thermal degradation product of agrocin 84 referred to as FNT. FNT is an antibiotic with which pAt-84a could be directly selected in genetic experiments. Using this selection and the range of transposons available in *A. radiobacter*, it should be possible to construct a mutant derivative of this plasmid that will no longer transfer to virulent recipients.

A third way of break down of biological control would result from the transfer of a Ti plasmid to strain 84. This possibility has not yet been investigated and no such transfers have been reported to have occurred in the field.

CHAPTER 6

AGROCINOPINES - THEIR DETECTION AND PROPERTIES

INTRODUCTION

Sensitivity to the nucleotide bacteriocin, agrocin 84 (Tate *et al.*, 1979), is one of the few known genetic markers for nopaline Ti plasmids (van Larebeke *et al.*, 1975; Watson *et al.*, 1975; Engler *et al.*, 1975). The basis of agrocin 84 specificity and sensitivity is a high affinity uptake system encoded by the Ti plasmid (Murphy and Roberts, 1979). Since it seems unlikely that agrocin 84, a toxic molecule, was the legitimate substrate for this permease, a non-toxic substrate was postulated. A search for such a substrate revealed a group of phosphorylated compounds that interact with agrocin 84. These compounds are found only in crown gall tumours incited by nopaline or agropine strains of *A. radiobacter*. Since these compounds conform to the definition of opines (Chapter 1), the name agrocinopines is proposed for these new crown gall specific metabolites.

The experiments described in this chapter involving uptake of [^{32}P] agrocin 84 were done collaboratively with P.J. Murphy.

MATERIALS AND METHODS

Bioassay for Agrocinopines

Plant tissue was macerated and extracted overnight in 70% ethanol (1ml/mg tissue). After centrifugation, the supernatant was

lyophilised or rotary evaporated at 35° and redissolved in 1/10th the original volume of 70% ethanol. The extract (20-40μl) was applied as a 1cm band to the centre of a piece of Whatman 3MM paper and subjected to electrophoresis in formic-acetic buffer, pH 1.7, for 20 min. at 65 V/cm. A strip was removed from the electrophoretogram and cut into $1cm^2$ pieces which were placed on plates containing agrocin 84 prepared by the method of Kerr and Htay (1974) (Chapter 2). Plates were then sterilised with chloroform vapour, overlaid with an indicator strain in soft agar then incubated at 27° for 24-36 h.

Detection reagents

Nopaline was detected using *phenanthrenequinone reagent* (Yamada and Itamo, 1966). Twenty mg of phenanthrenequinone are dissolved in 160ml of ethanol. To this, 40ml of aqueous 25% NaOH are added. The electrophoretogram is dipped in the reagent and dried in a cool air stream. Nopaline and other guanidines are detected as green fluorescent spots under longwave UV light.

p-anisidine reagent (Hough *et al.*, 1950)

5g of p-anisidine HCl is dissolved in 475ml of ethanol. To this is added 0.5mg of sodium metabisulphite in 25ml of H_2O. Electrophoretograms are dipped, dried and heated at 110° for 3-5 min.

Alkaline silver nitrate reagent (Trevelyan *et al.*, 1950)

The reagent is made by dissolving $AgNO_3$ in 20ml H_2O then adding 1 ℓ of acetone. Electrophoretograms are dipped through this reagent and dried at room temperature, then redipped in an alkaline reagent, made by adding 10ml of 20% NaOH to 90ml of ethanol. When the spots have appeared, electrophoretograms may be fixed by dipping

into a reagent made by dissolving 15g of sodium metabisulphite and 100g of sodium thiosulphate in 1ℓ of H_2O. The electrophoretograms are then rinsed with tap water and dried.

Phosphomolybdate reagent (Harrap, 1960)

50g of $Na_2MoO_4.2H_2O$ are dissolved in 250ml of H_2O. To this are added 500ml of 1N HCl and 210ml of 72% perchloric acid. The reagent is diluted 1:1 with H_2O. Electrophoretograms are dipped into a mixture of 40ml of this reagent and 160ml of acetone and heated for 15 min at 60°. They are then irradiated with short wave UV light. Phosphorus compounds appear as blue spots.

Weiss and Smith's reagent (Weiss and Smith, 1967)

Electrophoretograms are dipped into reagent A (1ml H_2O, 1ml of 50% periodic acid, 2ml pyridine and 100ml acetone). When dry they are redipped in reagent B (15g ammonium acetate, 0.3ml acetic acid, 1ml acetyl acetone in 100ml methanol). Yellow green fluorescent spots which become visible yellow spots, appear in 0.5 to 1 h.

Ketose reagent for sodium borate electrophoretograms (Frahn and Mills, 1959)

Electrophoretograms are sprayed with an aqueous solution of 0.5M urea and 1.0M tartaric acid and heated at 110°. Ketose sugars strain bright blue.

Buffers for electrophoresis and ion exchange chromatography

(1) Formic/acetic, pH 1.75

- 28.4ml of 99% formic acid and 59.2ml of acetic acid made up to 1ℓ with water.

(2) 0.05M citrate buffer, pH 5

- 10.5g citric acid, and 4.2g NaOH made up to 1ℓ with water.

(3) 0.1M ammonium bicarbonate buffer, pH 9.22

- 9.05 NH_4HCO_3 in 1ℓ of water adjusted to pH 9.22 with about 5ml of concentrated ammonia.

(4) sodium tetraborate buffer, pH 9.22

- 19.07g sodium tetraborate in 1ℓ of water.

(5) 0.1M sodium acetate buffer, pH 4.6

- 6g acetic acid/litre adjusted to pH 4.6 with about 10ml 20% NaOH.

(6) 0.1M triethylamine acetate buffer, pH 5.03

- 6.0g acetic acid and 7.02g of triethylamine made up to 1ℓ with H_2O.

(7) 0.3M triethylamine borate buffer, pH 9.4

- 15.38g of triethylamine and 18.64g of boric acid made to 1ℓ with H_2O.

<u>Determination of concentrations of agrocinopines</u>

Concentrations of agrocinopines A and B were determined in arabinose equivalents using a modified phloroglucinol-HCl reaction of Dische (1962). A standard curve was prepared using stock solutions of arabinose (0.2 - 1mg/ml); 50μl of each stock solution was added to

1 x 15cm tubes; then 5ml of a reagent (110ml of glacial acetic acid, 2ml of concentrated HCl and 4.5ml of 5% phluroglucinol in ethanol) was added and the tubes vortexed. The ingredients were boiled vigorously for 15 min, then cooled in tap water and the intensity of the pink colouration measured at 550nm. Dilutions of agrocinopines A and B were treated at the same time and concentrations read from the standard curve.

Concentrations of agrocinopines C and D which do not contain arabinose, were determined in glucose equivalents using the L-cysteine-sulphuric reaction (Dische, 1962). The procedure is as follows: to 1ml of a solution containing 10-100µg of hexose is added under cooling in iced water, 5ml of 86% sulphuric acid (1 part of water plus 6 parts of sulphuric acid). After 2 min, the sample is gently shaken in the iced water, then taken out and vigorously stirred, placed 1 min in tap water, and afterwards heated 3 min in a vigorously boiling water bath. After cooling in tap water, 0.1ml of a 3% solution of L-cystein hydrochloride monohydrate is added, and the sample is vigorously shaken. A yellow colour appears and the absorption is read at 412nm after several minutes. Dilutions of agrocinopines C and D were treated at the same time and concentrations were read from the standard curve.

Preparative isolation of agrocinopines

For biological and chemical characterisation of agrocinopines, it was necessary to have a large scale purification procedure. Agrocinopines were extracted from up to 1kg of tumour tissue by macerating the tumours in 70% ethanol with a kitchen blender and extracting

overnight at 4° with 70% ethanol (5ml/gm). The extract was then passed through 4 layers of cheesecloth and allowed to stand overnight at 4° to allow the finer particles to sediment. The extract was then decanted and rotary evaporated at 35° to remove the ethanol. The aqueous cloudy solution was left for a further 24h at 4° during which time the fine suspended material settled. The clear supernatant was carefully removed, filtered on a Buchner funnel through Whatman 541 paper, and passed through a Dowex 50 x 2 (H^{+}) column of dimensions 4cm x 60cm to remove cationic species (e.g. nopaline). The column was rinsed with 5 bed volumes of water and the effluent was then applied to a DEAE Sephadex A25 column (acetate form). Column dimensions were 4cm x 60cm. After washing away the neutral material with 5 bed volumes of H_2O, agrocinopines were eluted with 0.1M triethylamine acetate buffer, pH 5, pumped onto the column at 1ml/min. 15ml fractions were collected and those containing agrocinopines were detected with p-anisidine stain (Hough *et al.*, 1950) after electrophoresis of 5-10μl samples of every fifth fraction in formic/acetic acid, pH 1.7. The fractions containing agrocinopines were pooled and rotary evaporated. When a viscous residue remained, 200ml of H_2O was added and rotary evaporation was continued. This was repeated several times to evaporate much of the triethylamine. The residue was dissolved in water and applied to a column of DEAE Sephadex A25 (borate form) and eluted with 0.3M triethylamine borate, pH 9.4, which was also applied to 1ml/min. This procedure separated agrocinopine A from B and agrocinopine C from D. The fractions containing each agrocinopine were pooled and passed through a Dowex 50(H^{+}) column to remove triethylamine. The

Dowex column was rinsed with 5 bed volumes of H_2O and the total effluent was reduced in volume by rotary evaporation in a 2ℓ flask at 30° until the pH reached 3. Approximately 300ml of methanol was then added to the flask and rotary evaporated at 30° to remove the boric acid by conversion to volatile methyl borate. During this procedure, the agrocinopines were never taken to dryness in order to avoid acid hydrolysis as the pH of the solution was lowered due to the acidic nature of the agrocinopines. When no more methanol was distilling, more methanol was added and the process repeated until no more boric acid could be detected. Boric acid was detected by igniting a small volume of the methanol-agrocinopine solution on a watchglass. Boric acid causes a distinctly green flame. The boric acid free residues were carefully neutralised with 0.1M NaOH and evaporated to dryness.

Agrocinopines C and D were purified in the same way from tumours induced by the agropine strain A281.

Purification of agrocinopines for degradative studies

Agrocinopines prepared by ion exchange were loaded as a band at one end of a strip on 3MM paper and electrophoresed for 1 hr at 1500 volts in NH_4HCO_3 buffer, pH 9.2. They were detected by staining strips with p-anisidine cut from the sides of the electrophoretogram, then the agrocinopines were eluted with water. Purification by electrophoresis in formic-acetic buffer yielded a white solid after lyophilization. However, examination of this material showed that some breakdown of agrocinopine A to agrocinopine B took place during

purification. For this reason, purification in NH_4HCO_3 buffer was preferred.

Catabolic studies involving agrocinopines

(a) Disappearance of agrocinopines A, B, C and D from liquid media

Agrocinopines were added to a modified (0.5g K_2HPO_4/ℓ) medium of Petit and Tempé (1978) (Chapter 2). The phosphorus level was lowered to prevent interference with electrophoresis and with detection of agrocinopines. Sodium glutamate (0.2%) was added as a source of carbon and nitrogen. Agrocinopines A and B were added to give final concentrations of 4.2mM and 1.5mM arabinose equivalents respectively. The final concentrations of agrocinopines C and D were 4.2mM and 1.6mM glucose equivalents respectively. A bacterial cell suspension (5µl, 5×10^8 cells/ml) was added to 100µl of agrocinopine medium in a 1cm x 10cm capped tube. No bacteria were added to the control tube. The presence or absence of agrocinopines was assessed after 48 h incubation at 25°. The cultures were centrifuged and 20µl of the supernatant was electrophoresed in formic/acetic acid buffer. In later experiments 5µl was electrophoresed. Agrocinopines were detected by p-anisidine stain.

(b) Utilisation of agrocinopines A and B as sole source of carbon

To the liquid medium of Petit and Tempé (1978) agrocinopines were added as carbon source and $(NH_4)_2SO_4$ (2.0mg/ml) was added as nitrogen source. The final concentrations of agrocinopines A and B were 3.0mM and 3.8mM arabinose equivalents respectively. A washed bacterial cell suspension (20µl, 5×10^8 cells/ml) was added to 500µl of agrocinopine

medium. Growth was assessed visually after 24h as described by Firmin and Fenwick (1978) for agropine growth studies.

Acid and alkaline hydrolyses

50-100μl of agrocinopine solutions were placed in glass tubes and acetic acid or ammonia added to give a concentration of 1.5M. The tops of the tubes were sealed with a hot flame.

Glucose tests

Glucose was detected by spotting 2-5μl of samples whose pH was in the range of pH 4.6 to pH 7 onto 'clinistix' glucose detection sticks. A glucose oxidase linked colour reaction enables ready identification of D-glucose.

α-glucosidase treatment with agrocinopine A

To 10μl of a solution of agrocinopine A, 10μl of 0.3M phosphate buffer, pH 6.8, and 2μl of enzyme solution was added. A control without enzyme was also run. Sucrose was used as a test substrate to show the enzyme was active. The reaction mixtures were incubated overnight at 27°C and electrophoresed in sodium tetraborate buffer.

Bacterial alkaline phosphatase (Sigma III)

2μl of enzyme suspension was added to a solution of the substrate that had been adjusted to 0.1M NH_4OH.

Invertase

Samples were treated with invertase in 0.1M sodium acetate buffer, pH 4.6, for 45 min at 37°.

RESULTS

Detection of agrocinopines by bioassay

(a) Agrocinopines A and B from nopaline tumours

Crude extracts of normal and tumorous plant tissues were subjected to high voltage electrophoresis in formic acetic buffer, pH 1.7, and bioassayed to detect compounds that would increase or decrease the toxic effect of agrocin 84 on sensitive strains tested in the standard plate bioassay of Kerr and Htay (1974). In both normal and crown gall tomato tissue, a neutral compound was detected which counteracted the inhibitory effect of agrocin 84 (Figure 6-1). The substance was apparently unrelated to crown gall and was not further investigated. Of much more interest was an anionic species detected in crown galls that had been induced on tomato stems by the nopaline strains A208, C58, K27 and K108, but not detected in healthy tomato stem tissue nor in tomato galls induced by the octopine strains *K338, R10 and K305. This substance increased the toxic effect of agrocin 84 and extended the zone of inhibition (Figure 6-2); it had no detectable biological activity in the bioassay when agrocin 84 was absent. When the tumour extract was electrophoresed longer, this activity could be separated into two peaks. These compounds were referred to as agrocinopines A and B.

(b) Agrocinopines C and D from agropine tumours

When extracts of galls induced by the agropine strains Bo542 and its transconjugant strain A281 (Guyon *et al.*, 1980) were tested in

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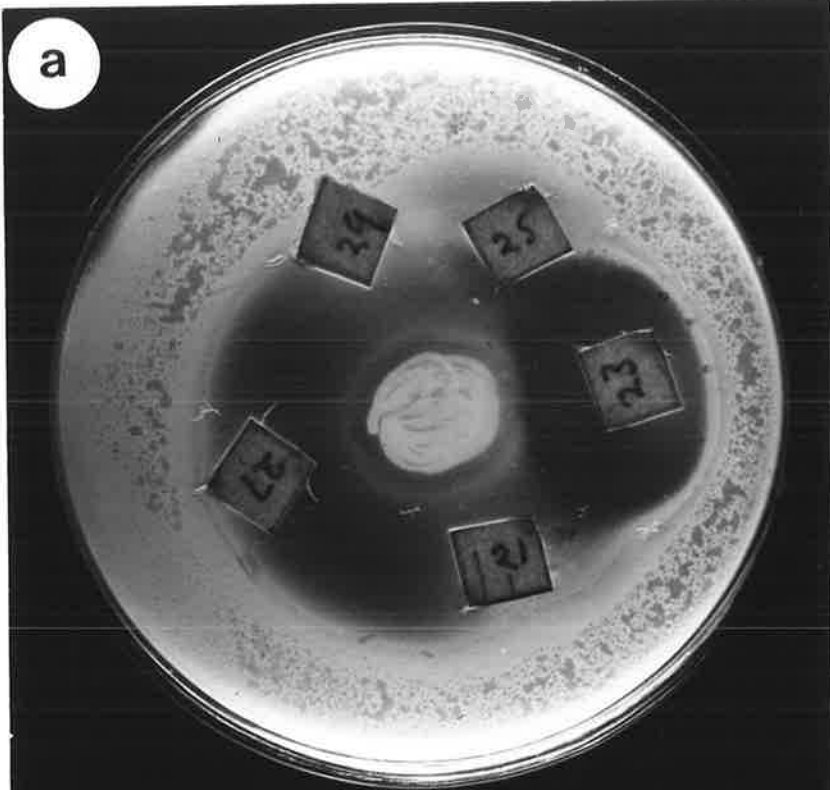
* K338 = C58Cl(pTi-1001)

Figure 6-1. Bioassay of total ethanolic extracts from normal tomato stem. 50µl of extract was added to a well cut in an agrocin 84 bioassay plate. When this extract was electrophoresed at pH 1.7, the material that counteracted agrocin 84 remained at the origin. During purification of agrocinopines this biological activity was associated with the effluent fraction that passed through the Dowex 50 (H^+) and DEAE Sephadex (acetate) columns.

Figure 6-2. Bioassay for agrocinopine A.

(a) bioassay of electrophoresed nopaline tumour extract. Agrocinopine A was detected in segment 23. Agrocinopine B was detected in segment 24 bioassayed on another plate.

(b) bioassay of agrocinopine A from DEAE Sephadex (acetate) column.


a

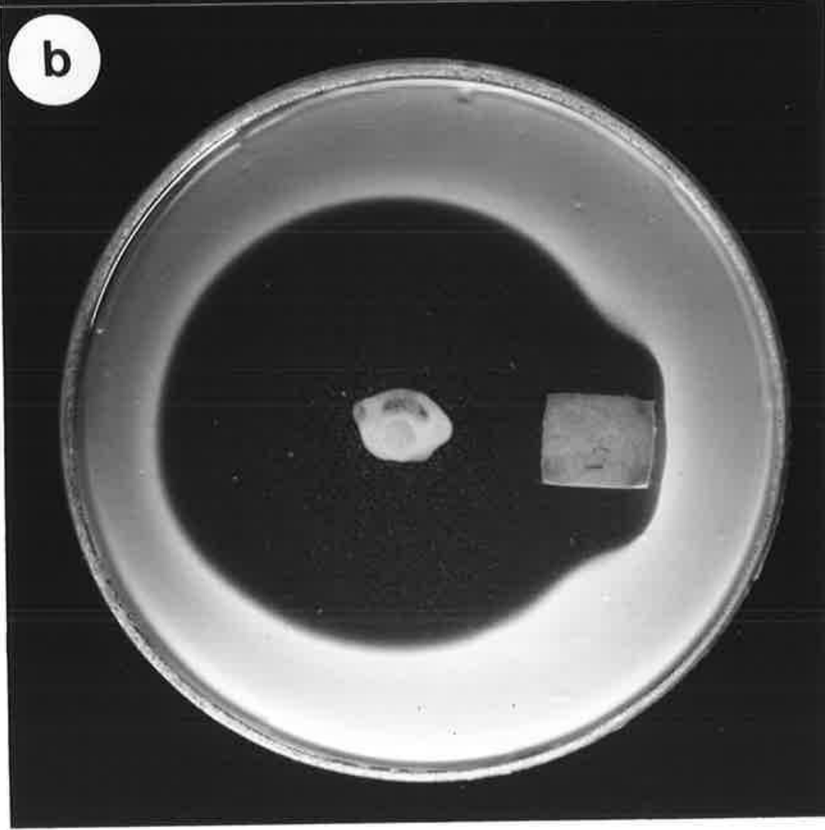
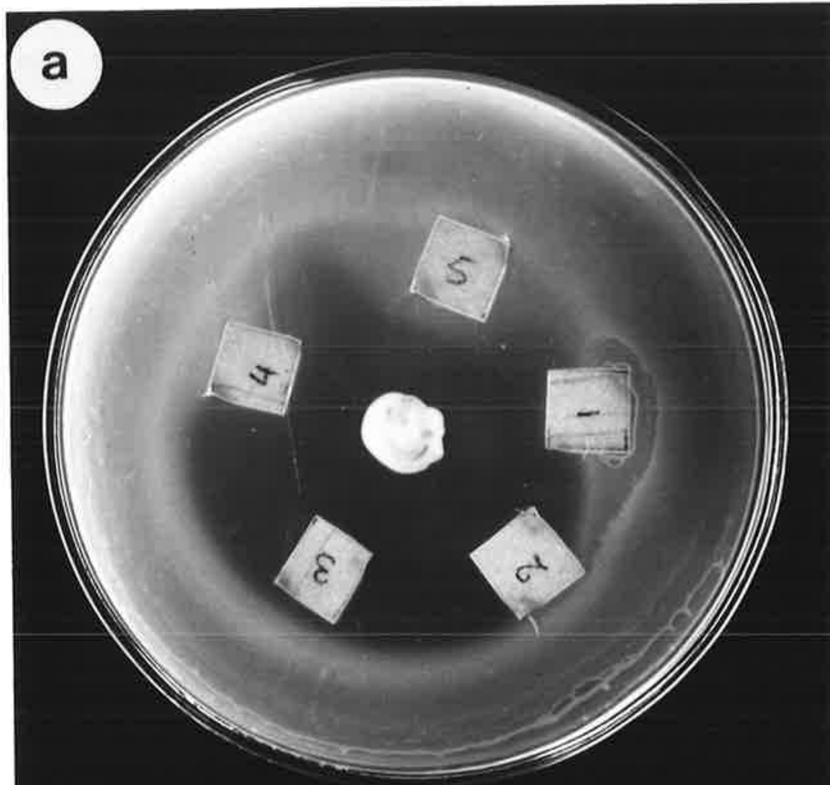

b

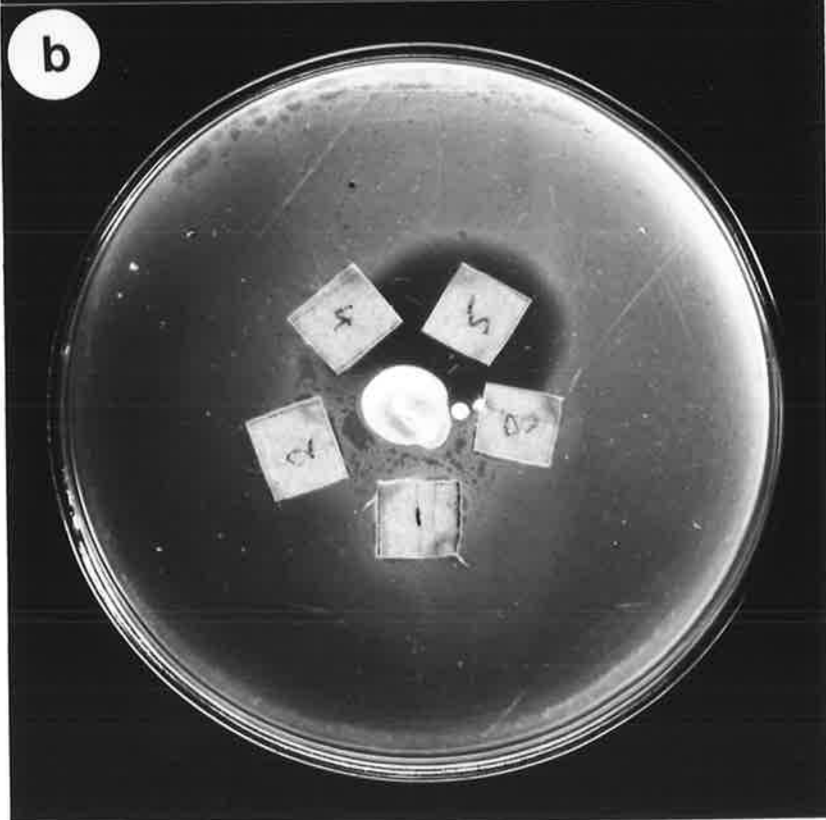
Figure 6-3. Bioassay for a mixture of agrocinopines C+D in electrophoresed extract of agropine tumour.

(a) agrocinopine C and D counteract the toxic effect of agrocin 84. The indicator is the nopaline strain A208.

(b) agrocinopine C and D induces sensitivity to agrocin 84 in the indicator strain A281.

Agrocinopine C and D were not clearly separated by the electrophoretic conditions used in this experiment (pH 1.7, 15 min 3000 V).


a


b

the same bioassay system, a further anionic compound, agrocinopine C, was detected that counteracted the toxic effect of agrocin 84 on the sensitive indicator strain A208 (Figure 6-3a). Furthermore, when the agrocin 84 insensitive strain A281 was used as the indicator, a zone of inhibition appeared where agrocinopine C had been added (Figure 6-3b). Biological activity was not detected when agrocin 84 was absent. Therefore, the biological activity detected in the bioassay must result from an interaction between agrocinopine C and agrocin 84. The presence of agrocinopine C made the agropine strain A281 sensitive to agrocin 84. During further purification of this compound, it was found to have two components, the second of which is referred to as agrocinopine D.

Agrocinopines in axenic tumour cultures

The biological activity of agrocinopines A and B was found to be associated with red-brown p-anisidine staining spots on electrophoretograms which were absent on normal plant extracts. Similarly, agrocinopines C and D were associated with yellow staining material. This stain and the bioassay technique was used to survey several axenic tumour cultures for the presence of agrocinopines.

Agrocinopines A and B were detected in a sterile tissue culture of tobacco crown gall induced by the nopaline strain T37. Agrocinopine C was detected in a tissue culture of Bo542 tobacco tumour. No agrocinopines were detected in sterile tissue cultures of tobacco crown gall induced by the octopine strain A66. The presence of living

bacteria is obviously not necessary for agrocinopine biosynthesis implying that the genetic information for expression of this trait is coded for by the T-DNA of nopaline and agropine Ti plasmids.

Agrocinopine synthesis by a Nos$^-$ mutant

Tumours induced on tomato stems by the Nos$^-$ (nopaline synthesis minus) insertion mutant GV3575 were examined. This strain was supplied by J. Schell, Ghent and has a Tn7 insertion in part of the T-DNA that directs nopaline synthesis. The tumours contained agrocinopines A and B but no nopaline.

Chemical properties of agrocinopines

The agrocinopines are anionic at both pH 9.22 and pH 1.75. Their anionic character at pH 1.75 indicates the presence of a strong acid. They are phosphorylated compounds and can be detected in paper electrophoresis with phosphomolybdate reagent. They can also be detected with alkaline silver nitrate and with the terminal glycol reagent of Weiss and Smith (1967). Agrocinopines A and B stain red brown with p-anisidine sugar reagent, whereas agrocinopines C and D stain yellow. Agrocinopines A, B and C can be detected after borate electrophoresis with the ketose sugar reagent of Frahn and Mills (1959). The electrophoretic and chromatographic mobilities of the agrocinopines are given in Table 6-1.

Degradative studies of the agrocinopines have given preliminary information on their chemical structures. Mild acetic acid hydrolysis

Table 6-1. Relative mobility values for the agrocinopines in several chromatographic systems.

Electrophoretic systems	Agrocinopines: A	B	C	D
*Formic-acetic pH 1.7 ≁ M_{OG}=	0.45	0.57	0.43	0.52
*0.05M sodium citrate pH 5 M_{OG}=	0.51	0.63	0.52	0.61
**0.1M NH_4HCO_3 pH 9.2 M_{OG}=	0.53	0.69	0.53	0.60
**0.05M NaB_4O_7 pH 9.2 M_{OG}=	0.63	0.97	0.55	0.73
Paper chromatographic system				
Ethyl acetate : pyridine : water				
10 : 4 : 3 Rf=	0.10	0.14	0.09	0.12

≁ OG is orange G, anionic reference dye with mobility = 1.

* non-migrating marker is fructose.

** non-migrating marker is 1 deoxyadenosine.

(1.5M, 110°, 15 min) of agrocinopine A yielded agrocinopine B and a hexose sugar identified as glucose by comparison with authentic glucose in sodium borate electrophoresis and ethyl acetate : pyridine : H_2O (10 : 4 : 3) paper chromatography. Its sensitivity to D-glucose oxidase indicated that this sugar was D-glucose. Longer acetic acid hydrolysis of agrocinopine A (1.5M, 110°, 60 min) yielded inorganic phosphate and a pentose sugar identified as arabinose by paper chromatography and by sodium borate electrophoresis. Acid hydrolysis of agrocinopine B (1.5M, 110°, 60 min) yielded arabinose and inorganic phosphorus. No glucose was detected.

Similar degradative studies have been carried out on agrocinopines C and D. Agrocinopine C was converted to agrocinopine D by mild acetic acid hydrolysis (1.5M, 110°, 15 min) indicating a close structural relationship between these two compounds. Longer (1.5M, 110°, 40 min) acetic acid hydrolysis of both agrocinopines C and D yielded D-glucose and inorganic phosphorus.

Agrocinopines A, B and C react with the ketose reagent described above. No ketose monosaccharides were detected in the acid hydrolysates presumably because ketose sugars are labile in acid conditions. However, alkaline degradation of agrocinopines A and C (1.5M NH_4OH, 110°, 40 min) yielded a ketose sugar with the identical relative mobilities of sucrose in 0.05M sodium borate, and 0.1M NaOH electrophoresis and in ethyl acetate : pyridine : H_2O (10 : 4 : 3) paper chromatography. Invertase treatment of this sugar yielded glucose and fructose. Therefore, it was concluded that agrocinopine

A contains glucose, fructose, arabinose and phosphorus and agrocinopine C contains glucose, fructose and phosphorus.

Apart from sucrose, alkaline degradation of agrocinopines yielded further products. When the products of ammonolysis of agrocinopine A were separated by electrophoresis on 0.1M NH_4HCO_3 buffer, pH 9.2, 3 anionic fragments were detected. Fragment 1A (M_{OG} = 1.0) was detected with phosphomolybdate reagent and alkaline silver nitrate reagent but not with p-anisidine stain. Fragments 2A and 3A (M_{OG} = 1.25 and 1.31 respectively) were phosphorylated and stained pink with p-anisidine. These fragments were eluted together and treated with bacterial alkaline phosphatase. The mixture of fragments 2A and 2B and the enzyme treated material was electrophoresed in formic/acetic acid buffer, pH 1.75. Fragments 2A and 2B both ran to the same position (M_{OG} = 0.68). Alkaline phosphatase treatment gave complete breakdown of these products yielding a pink staining neutral species. Although this product was not identified, the results from the acid hydrolysis of agrocinopine A imply that this neutral material is arabinose and the electrophoretic behaviour of fragments 2A and 3A and their sensitivity to alkaline phosphatase is evidence that they are isomers of arabinose phosphate.

When products of ammonolysis of agrocinopine C were separated by electrophoresis on NH_4HCO_3 buffer, 2 anionic fragments were detected. Fragment 1C (M_{OG} = 0.91) was phosphorylated and was detected with alkaline silver nitrate reagent but not p-anisidine. Fragment 2C (M_{OG} = 1.19) was phosphorylated and stained yellow with p-anisidine. In formic/acetic acid buffer fragment 2C had an electrophoretic mobility of 0.61. It was degraded by alkaline phosphatase and yielded D-glucose. The evidence is that fragment 2C is an isomer of glucose phosphate.

From the degradative studies outlined above it is evident that there is a structural similarity between agrocinopines A and C. They are both phosphorylated compounds containing sugars and after alkaline degradation both yield sucrose and sugar monophosphates. The most obvious difference between the two is that agrocinopine A contains arabinose.

Opine nature of agrocinopines

The characteristics of an opine have been defined in Chapter 1. Experiments were designed to determine whether the agrocinopines are opines. Three transconjugant strains were used; all had the genetic background of strain C58Cl but each contained a different Ti plasmid: a nopaline Ti plasmid (pTi-T37), an agropine Ti plasmid (pTi-Bo542) or an octopine Ti plasmid (pTi-1001). These strains were tested for inducing the synthesis of agrocinopines in tomato stem crown gall tissue. The results are presented in Table 6-2.

The same strains were used to study agrocinopine catabolism. Two methods were used: the first involved monitoring the disappearance of agrocinopines from liquid media containing 2mg/ml sodium glutamate as a source of carbon and nitrogen (Table 6-2). In the second method agrocinopines A and B were tested as sole sources of carbon.

The results (Table 6-2) indicate that the biosynthesis of agrocinopines A and B is specified by the nopaline Ti plasmid and the biosynthesis of agrocinopines C and D by the agropine Ti plasmid.

The results from the catabolic studies are more complex. When sodium glutamate is present as the source of carbon and nitrogen, all strains slowly converted agrocinopine A to agrocinopine B so that both opines were present in the medium at 48 h. Since the Ti-plasmidless strain C58Cl can bring about this conversion, it is not a Ti plasmid coded character. This conversion can also be brought about by mild acid hydrolysis (see above) and by treatment with α-glucosidase. Agrocinopines A and B were completely catabolised only by strain A208 which harbours a nopaline Ti plasmid. Only this strain was able to grow on agrocinopine A and B as sole source of carbon. (Strain C58 was not tested.)

In all experiments, agrocinopine C was used by the agropine strain A281. In some experiments agrocinopine C was catabolised by the nopaline strains A208 and C58 and in others it was not. Therefore, in one experiment the nopaline strains C58, A208 and K122 were incubated longer (for 72 hours) in medium containing agrocinopine C. Strains A208 and K122 used all the agrocinopine C while a trace remained in the tube inoculated with C58. In the same experiment, C58Cl and the octopine strain K338 did not use agrocinopine C. It was concluded that the nopaline strains can use agrocinopine C but perhaps at a slower rate than the agropine strain A281 which used this opine in every experiment. Agrocinopine D was catabolised in all experiments by A281, and the nopaline strains A208, C58 and K122, but never by C58Cl or the octopine transconjugant strain K338.

Table 6-2. Ti plasmid specific synthesis and catabolism[a] of agrocinopines.

Strain	Ti plasmid[c] type	Agrocinopines in tumours	Agrocinopines catabolism A	B	C	D
C58Cl	-	avirulent	[d]A→B	-	-	-
A208[b]	nopaline	A+B	+	+	-	+
A281[b]	agropine	C+D	A→B	-	+	+
K338[b]	octopine + agropine	-	A→B	-	-	-
C58	nopaline	A+B	+	+	-	+

a Determined by disappearance of agrocinopines from the liquid medium.

b Transconjugants in C58Cl background.

c Refers to whether octopine, nopaline or agropine are synthesised by tumours induced by these strains.

d Agrocinopine A converted to agrocinopine B in culture medium but not in uninoculated controls.

Agrocin 84 resistant mutants and agrocinopine catabolism

Agrocin 84 is taken up by sensitive cells by a Ti plasmid coded permease (Murphy and Roberts, 1979). Mutants selected for resistance to agrocin 84 no longer take it up (P. Murphy, unpublished). If agrocinopines A and B enter the cell through the same permease, agrocin 84 resistant mutants should not take up agrocinopines. This was investigated. Several agrocin 84 resistant (Agr^r) mutants were tested. Strain A623 is an agrocin 84 resistant mutant derived from strain A208. Its Ti plasmid had undergone a deletion of 20×10^6 daltons (M.-D. Chilton, personal communication). The parental strain catabolised agrocinopines A and B but the mutant strain did not. Strain K57A Agr^r was also tested. This strain was derived from strain K57A.* No difference in electrophoretic mobility in the mutant and parental Ti plasmid could be detected even after prolonged electrophoresis of plasmid extracts in 0.7% agarose (P.J. Murphy, personal communication), so it must be either a point or a very small deletion mutant of the agrocin sensitivity region. The agrocin 84 resistant strain did not catabolise agrocinopines A and B. The parental strain did. A third pair of strains gave equivalent but slightly more complex results. C58C1(pGV3103) is an agrocin resistant mutant with Tn7 inserted into the Agr^r (agrocin sensitivity) locus. It catabolised neither agrocinopine A nor B. Its agrocin 84 sensitive sibling strain C58C1(pGV3181) with a Tn1 insertion in a 'silent' region of the T-DNA catabolised agrocinopine B but not A.

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* K57A = K57(pTi-27).

Interaction between agrocin 84 and the agrocinopines

The bioassay with which I first detected the agrocinopines indicates that an interesting interaction occurs between these opines and agrocin 84. This interaction was studied in more detail by investigating the effect of agrocinopines on uptake of ^{32}P-labelled agrocin 84 by strains of *A. radiobacter*.

^{32}P-labelled agrocin 84 was prepared and uptake studies carried out as described by Murphy and Roberts (1979) and Chapter 2. The cells were pre-incubated in the presence of agrocinopine A for 6 hr and then washed once before uptake experiments. Agrocinopine A and [^{32}P]agrocin 84 were added simultaneously when the uptake of agrocin in the presence of agrocinopine A was being assessed.

Preincubation of the agrocin-sensitive nopaline strain K57A with agrocinopine A greatly increased the rate of uptake of agrocin 84 (Figure 6-4a). Agrocinopine B was not tested.

The agropine strain A281 is normally insensitive to agrocin 84 and does not take up [^{32}P]agrocin 84 (Figure 6-4b). When it was preincubated with agrocinopine C it did take up agrocin 84 (Figure 6-4b) although to an extent less than did the nopaline strain K57A. Agrocinopine D was not tested.

Figure 6-4a also shows the effect of the presence of agrocinopine A on agrocin 84 uptake. When agrocinopine A and agrocin 84 were added simultaneously to strain K57A, agrocin 84 uptake was blocked.

Figure 6-4. Effect of agrocinopines on uptake of [^{32}P] agrocin 84.

(a) The effect of agrocinopine A on the uptake of [^{32}P]agrocin 84. (2×10^{-8}M, 35,000 cpm) by strain 57A.

●—● strain K57A preinduced for 4 hr with 4.2mM arabinose equivalent of agrocinopine A.

▲—▲ uninduced strain K57A to which agrocinopine A and [^{32}P]agrocin 84 were added simultaneously just before the experiment.

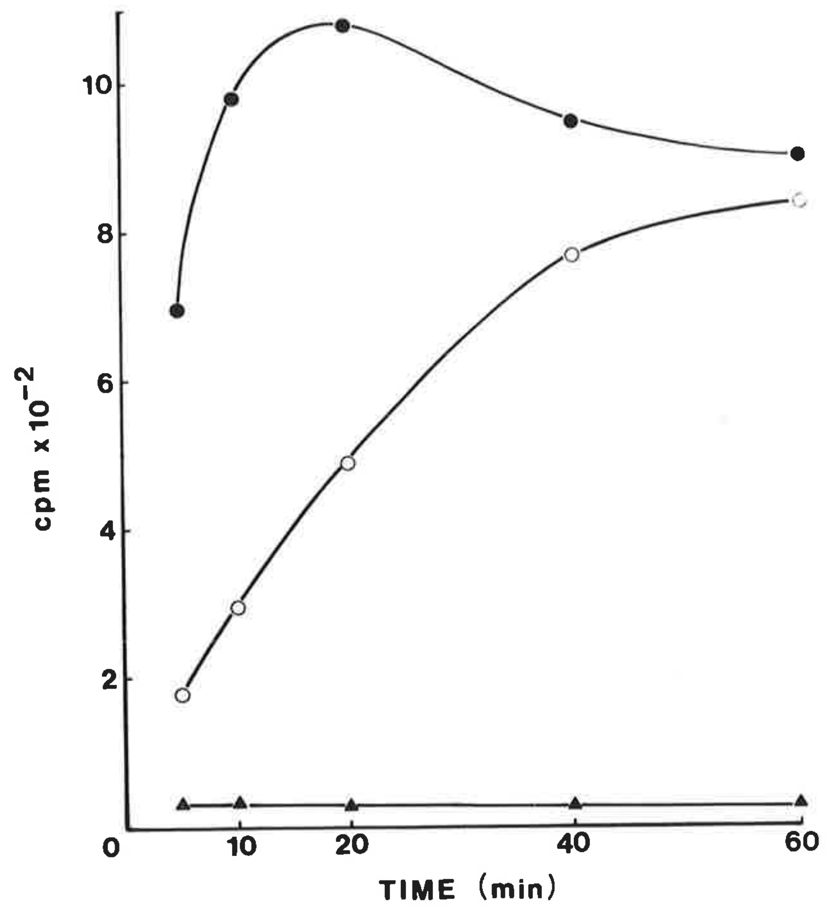
o—o strain K57A uninduced control.

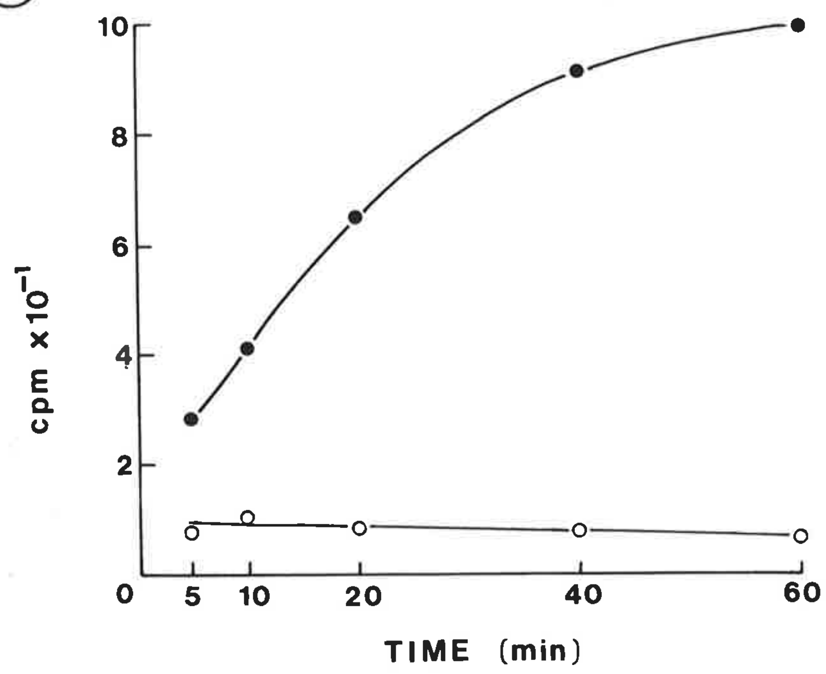
The bacteria were resuspended at OD_{680} = 0.17.

(b) The effect of agrocinopine C on uptake of [^{32}P] agrocin 84 (2.4×10^{-8}M, 46,000 cpm) by strain A281.

●—● strain A281 preinduced for 6 hr with 4.2mM glucose equivalents of agrocinopine C.

o—o strain A281 uninduced.


a
cpm x 10^{-2}
10
8
6
4
2
0
10
20
40
60
TIME (min)


b
cpm x 10^{-1}
10
8
6
4
2
0
5
10
20
40
60
TIME (min)

DISCUSSION

Agrocinopines are the first reported phosphorylated opines. They are referred to as opines because they share several biological properties with previously discovered opines such as octopine and nopaline. First, agrocinopine biosynthesis in crown gall tissue depends on the type of Ti plasmid in the pathogen. Secondly, these compounds are synthesised in sterile crown gall tissue cultures and have not been detected in normal plant tissue. The presence of the living bacterium is clearly not necessary for their continued synthesis. This fact indicates that the biosynthesis of agrocinopines may be directed by T-DNA.

Different genes must be involved in the synthesis of nopaline and agrocinopines A and B because these agrocinopines were detected in tomato crown gall tissue induced by a mutant nopaline strain that is unable to induce nopaline synthesis in tumours. It should now be possible to define three independent functional units of the nopaline Ti plasmid linked T-DNA, namely regions involved in oncogenicity (Depicker *et al.*, 1978; Holsters *et al.*, 1980), nopaline synthesis (Holsters *et al.*, 1980) and agrocinopine synthesis. It should be possible to define the agrocinopine synthesis region with transposon insertion mutants mapped in the 'silent' regions of the T-DNA of the Ti plasmid, that is, those regions of T-DNA where transposon insertions affect neither virulence nor nopaline synthesis (Holsters *et al.*, 1980).

The third property that agrocinopines share with the previously described opines is that their catabolism is specified by the Ti plasmid.

Agrocinopines A and B can be used as sole carbon source only by strains harbouring a nopaline Ti plasmid. Although insufficient material was available to test agrocinopines C and D as sole sources of carbon, I have shown that these opines disappeared from liquid media inoculated with strains carrying certain Ti plasmids. In all experiments, agropine strain A281 catabolised agrocinopine C. Agrocinopine C was also used by nopaline strains although the results were sometimes more complicated. For example, in some experiments agrocinopine C was used by the nopaline strain A208, yet not in others. It is possible that nopaline strains use agrocinopine slower than do agropine strains. Agrocinopine D was also catabolised by agropine and nopaline strains. It can be concluded that the presence of a nopaline or agropine Ti plasmid was necessary for catabolism of these two opines.

During experiments in which the disappearance of agrocinopine A from liquid medium was being monitored I found that agrocinopine A was slowly converted to agrocinopine B in the presence of the Ti plasmidless strain C58CI. This conversion which involves the loss of D-glucose can also be brought about enzymically by α-glucosidase or chemically by mild acetic acid hydrolysis. A close structural relationship, therefore, exists between agrocinopines A and B. With the present data, it is not possible to say whether both these compounds are synthesised in crown gall tissue or whether agrocinopine B results from the degradation of A. Mild acid hydrolysis of agrocinopine C also produces agrocinopine D. So the same reasoning applies to this pair of opines. The resolution of these questions will require complete elucidation of structures and

identification of precursors so that biosynthetic studies with labelled precursors can be carried out.

Agrocinopines were initially discovered because it seemed unlikely that the agrocin 84 permease (Murphy and Roberts, 1978) existed to transfer this toxic molecule into cells of *A. radiobacter*. Are agrocinopines the legitimate substrates for this permease? Several facts argue that they are. The first is that preincubation of the noplaine strain K57A with agrocinopine A increased the rate of uptake of agrocin 84 (Figure 6-4a). This observation supports the hypothesis that the agrocin 84 permease is normally partially expressed and that it is fully induced by agrocinopine A. The second is that agrocin 84 resistant mutants no longer catabolise agrocinopines A and B; nor do they take up agrocin 84 (P.J. Murphy, personal communication). One agrocin 84 resistant mutant that was tested carried the transposon Tn7 in the *agr* (agrocin sensitivity) gene. It did not catabolise agrocinopine B. Its agrocin 84 sensitive sibling strain which carried Tnl inserted into the T-DNA did. However, neither of these two strains catabolised agrocinopine A. This anomalous behaviour of the agrocin sensitive sibling strain has not yet been investigated further.

The third fact is that the presence of agrocinopine A blocked agrocin 84 uptake (Figure 6-4). Although all the observations argue in favour of agrocinopines being the natural substrates for the agrocin 84 permease, formal proof will need further uptake studies to determine whether agrocinopines act as competitive inhibitors of agrocin 84 uptake.

An unexpected outcome of these experiments was that sensitivity of the agropine strain A281 to agrocin 84 can be induced

by agrocinopine C. I am unable to explain why this strain takes up agrocin 84 only after growth with agrocinopine C while the agrocin 84 permease of the nopaline strains is partially expressed.

CHAPTER 7

STUDIES ON CONJUGAL TRANSFER OF Ti PLASMIDS

The Ti plasmids are conjugative plasmids (Chilton *et al.*, 1976; Kerr and Roberts, 1976; van Larebeke *et al.*, 1977; Kerr *et al.*, 1977; Genetello *et al.*, 1977; Montoya *et al.*, 1977; Hooykaas *et al.*, 1977). The conjugative properties of the octopine Ti plasmids have been most extensively studied. These investigations revealed that conjugative activity is normally repressed and can be induced by octopine, lysopine and octopinic acid (Petit and Tempé, 1978; Tempé *et al.*, 1978a; Klapwijk *et al.*, 1978), all members of the pyruvic opine group (Guyon *et al.*, 1980). Octopine also induces the genes involved in octopine catabolism and the genetic data support a model of negative control by a single repressor of two separate operons controlling octopine catabolism and Ti plasmid transfer (Petit *et al.*, 1978a; Klapwijk *et al.*, 1978; Klapwijk and Schilperoort, 1979).

Transfer of nopaline and agropine Ti plasmids has not been extensively studied. Some reports claimed that nopaline induces transfer of nopaline Ti plasmids (Kerr *et al.*, 1977; Petit *et al.*, 1978a; Hooykaas, 1979).

Hooykaas (1979) studied the effect of nopaline on Ti plasmid transfer from several nopaline strains including T37. This strain transferred its Ti plasmid at the frequency of 10^{-4} per recipient only after preculture in the presence of nopaline. He selected five mutants

of this strain for growth on octopine as sole source of nitrogen and found that these mutants transferred their Ti plasmid without induction by nopaline and proposed a model of 'coorindated regulation' of transfer and nopaline catabolism. However, he and Petit *et al.* (1978a) found that the transfer of the nopaline Ti plasmid of strain C58 was not induced by nopaline. Furthermore, Petit *et al.* (1978a) reported that transfer of the Ti plasmid of K14 occurred as frequently with or without preculture of K14 in the presence of nopaline. The anomalies of the transfer behaviour of nopaline Ti plasmids and the role of agrocinopines in this process are studied in this chapter.

The agropine Ti plasmids have only recently become amenable to genetic studies. Guyon *et al.* (1980) showed that Ti plasmid transconjugants could be selected on agropine but that the transfer frequency was very low. The transfer properties of this group of Ti plasmids are also investigated in this chapter.

Ti plasmids that encode bacterial octopine catabolism and tumour cell octopine synthesis are found in biovar 1 and 3 strains. The biovar 1 octopine plasmids form a uniform group as judged by their SmaI restriction digest patterns (Sciaky *et al.*, 1977). The biovar 3 Ti plasmids give rise to a completely different restriction pattern (Chilton *et al.* (in press); Thomashow *et al.*, 1980b). The biovar 1 octopine strains also induce agropine synthesis (Firmin and Fenwick, 1979; Ellis and Tate, unpublished results).

Experiments described in this chapter were carried out to investigate whether the transfer functions of a biovar 1 octopine Ti plasmid which are induced by octopine are also induced by agropine, whether transfer functions of biovar 3 Ti plasmids are induced by octopine and whether biovar 3 strains induce tumours that synthesise agropine.

Some of the preliminary tests on transfer induction by nopaline described in this chapter were done in collaboration with Allen Kerr and Jacques Tempé. Synthetic nopaline and the agropine analogue, mannopine, were synthesised by Jacques Tempé and were invaluable in carrying out selection for nopaline and agropine Ti plasmids.

MATERIALS AND METHODS

Bacterial Strains Used as Donors

Where possible, donor strains were used that were isolated independently from different areas so that as wide a range of strains as possible was tested. Details of these strains appear in Table 2-1.

Media

The minimal salt medium of Petit and Tempé (1978) (Chapter 2), was used in conjugation and selective media. Selective medium for nopaline plasmids contained 2mg/ml nopaline as sole source of carbon and nitrogen, 100µm/ml rifampicin and 500µg/ml streptomycin. For

biovar 2 strains 0.2μg/ml biotin was added. Selective medium for agropine Ti plasmids contained 2.0mg/ml $(NH_4)_2SO_4$, 2mg/ml 'Mannopine' as sole source of carbon, 100μg/ml rifampicin and 500μg/ml streptomycin.

Chemicals

Nopaline, synthesised by J. Tempe using the method of Cooper and Firmin (1977), was used. The natural isomer was separated from allo-nopaline by re-crystallisation from an aqueous solution.

Natural nopaline was extracted from crown gall tissue as described by Petit and Tempé (1978).

'Mannopine' was prepared from glutamic acid and mannose as described by Tempé *et al.* (1980b).

Agrocinopines were isolated as described in Chapter 6. Concentrations of agrocinopines were determined as described in Chapter 6.

Conjugation

(a) Replica plating method. Strains were initially screened for transfer induction by nopaline using a replica plating method. On day one, donors were applied as patches to agar plates containing nopaline (2mg/ml) as sole source of carbon and nitrogen (opine medium) or to plates containing 2mg/ml sodium glutamate for biovar 1 donors or plates containing 2mg/ml sodium glutamate and 2mg/ml sodium tartrate for biovar 2 strains (non-opine media). (Biovar 2 strains did not grow well on sodium glutamate alone.) On day two, a recipient

(C58CIRifStr or K57RifStr) was spread on selective medium. On day three, donors were replica plated onto the recipient lawn on the selective plate. These plates were incubated at 27°C for 7 days.

(b) Drop mating method. On day one, the recipient strain from a YM agar slope was suspended in water and spread onto selective medium. Only half of the plate was inoculated. At the same time, the donors were resuspended from YM agar slopes and 10µl of the suspension inoculated onto 100µl of agar in 10 cm x 1 cm glass tubes. The agar contained $(NH_4)_2SO_4$ (2mg/ml), with mannitol (1mg/ml) in the non-opine medium, and nopaline (2mg/ml), agrocinopine A (5mM arabinose equivalents) or agrocinopine C (20mM glucose equivalents) in the opine media. The minimum level of agrocinopines needed to induce transfer have not been determined. In later experiments, to ensure good growth of the donors, all tubes contained 2.0mg/ml $(NH_4)_2SO_4$ and 1.0mg/ml mannitol which were supplemented with the same concentration of opines as before. On day two, the donors were resuspended in 200-500µl of sterile water and 10µl were spotted onto the selective plates. The first drop from each tube was applied to the uninoculated half of the plate to assess whether the medium counterselected the donor and the second drop applied to the lawn of recipient. The drops were allowed to dry and the plates were incubated at 25°C.

(c) Quantitation of donor efficiency. The drop mating method was used. Donors were diluted by 10 to 10^{-7}. 10µl of the 10^{-7}, 10^{-6}, 10^{-5} dilutions were spotted onto agar containing 2.0mg/ml $(NH_4)_2SO_4$ and 1.0mg/ml mannitol to determine the viable count. 10µl of the 10^{0}

dilution was spotted onto selective medium as a control and 10µl of the 10^{0} to 10^{-4} dilutions were spotted in triplicate onto a lawn of the recipient growing on selective medium. Donor efficiencies were calculated as the number of transconjugants observed per donor cells applied.

Phage typing

Transconjugants were purified on selective medium and then tested for phage type. Donors and recipients were tested at the same time. Where donor and recipient had the same phage type, e.g. C58 and C58C1, another recipient, K57RifStr was also used to dispel any ambiguity that transfer had been induced.

Detection of agropine in tumours

Tumour tissue was extracted and electrophoresed as described in Chapter 6. Agropine was detected with alkaline silver nitrate reagent (Trevelyan *et al.*, 1950).

RESULTS

A. Nopaline Strains

The strains harbouring nopaline Ti plasmids listed in Table 7-1 were used as Ti plasmid donors in conjugation experiments. The strains K84 and K112 were also used as donors. These are examples of naturally occurring non-oncogenic strains of *A. radiobacter* that harbour nopaline catabolic (NOC) plasmids. All strains were tested initially

Table 7-1. Plasmid transfers from nopaline strains.

Strain	Plasmid transfer after pre-culture on:		
	non-opine medium	nopaline	agrocinopine A
K11	+	+	NT
K14	+	+	NT
K19	-	-	+
K31	-	-	+
K120	+	+	NT
K122	-	-	+
IIBV7	-	-	-
C58*	-	-	+
H100	-	-	+
T37	+	+	+
K27	-	-	+
K105	-	-	+
K108	-	-	+
Ag43	-	-	+
K84	-	+	+
K112	-	+	+

NT Not tested.

* pTi-C58 has also been induced to transfer by preculture of C58 on 15mM arabinose equivalents of agrocinopine B as sole carbon source.

by the replica plating technique and then by the drop method. Strain C58ClRifStr was used extensively as the recipient strain. Strain K57RifStr was also used in several experiments and gave the same results as strain C58ClRifStr. Results of these conjugation experiments are summarised in Table 7-1. Most strains transferred their Ti plasmids only after induction with agrocinopine A. Agrocinopine B also induced the transfer of pTiC58 but was not tested as an inducer for the other strains. Strains K11, K14, K120 and T37 did not require induction by opines for transfer. The NOC plasmids of K84 and K112 (referred to as pAt-84b and pAt-112a respectively) transferred after preincubation on either nopaline or agrocinopine A. The only strain that did not transfer its Ti plasmid was IIBV7. Transconjugants of Ag40 appeared very slowly and unlike Ag40 grew very slowly on nopaline medium.

In a further experiment, the biological activities of synthetic nopaline and natural nopaline isolated from crown gall tissue were compared. Natural nopaline was tested at 10mM using the drop conjugation technique. It induced the transfer of the NOC plasmids pAt-84b and pAt-112a, but not the transfer of nopaline Ti plasmids. Therefore the biological activity of natural and synthetic nopaline is identical.

Donor efficiencies of nopaline Ti plasmids

Two strains were chosen to determine donor efficiencies of nopaline Ti plasmids. C58 was an example of a strain that transfers only after induction with agrocinopine A (or B). T37 was chosen as

an example of a strain that transfers without induction by an opine. The results of these experiments are given in Table 7-2. Although T37 transferred its Ti plasmid without induction, preculture in the presence of agrocinopine A greatly increased transfer frequency.

Transfer of Ti plasmids constitutive for nopaline catabolism

Mutants constitutive for nopaline catabolism (Noc^{c}) can be isolated by plating on octopine, a non-inducing substrate of the nopaline catabolic pathway. Noc^{c} mutants of C58 can also be isolated by plating on arginine as carbon source which is also a non-inducing substrate of enzymes in the nopaline catabolic pathway (Chapter 3). This latter method is only applicable to strains that do not have an alternative non-Ti plasmid linked arginine catabolic pathway.

Four independent Noc^{c} mutants of C58 selected on arginine were tested for transfer. They all required preinduction with agrocinopine A. Five more mutants of C58 were selected for growth on octopine as carbon and nitrogen source. None was constitutive for transfer. Several Noc^{c} mutants of each of the strains K27, K108 and Ag40 were tested for transfer. Again, none was constitutive for transfer. Similar mutants of K31 and H100 also did not transfer their Ti plasmid constitutively (A. Kerr, personal communication).

B. Transfer of Agropine Ti Plasmids

Only a few examples of this class of plasmid are known. These are the plasmids that were previously called 'null' type before any opine products had been discovered in galls induced by these strains.

Table 7-2. Per donor Ti plasmid transfer frequencies for C58 and T37.*

Strain	2.0mg/ml $(NH_4)_2SO_4$ + 1mg/ml mannitol	2mg/ml nopaline	2.0mg/ml $(NH_4)_2SO_4$ + 1mg/ml mannitol + Agrocinopine A
C58	0	0	1.6×10^{-2}
T37	1.2×10^{-5}	5×10^{-5}	1.8×10^{-1}

* In a further experiment, pTi-T37 transferred at 7×10^{-4} and 1×10^{-4} after pregrowth of strain T37 on nopaline (2mg/ml) & sodium glutamate (2mg/ml) respectively.

Guyon *et al.* (1980) have shown that this class of plasmids induces galls that synthesise agropine and that bacteria harbouring these plasmids can use agropine as a growth substrate. These authors showed that agropine could be used to select for transconjugants of this plasmid class. However, transfer was infrequent which indicates agropine is not an inducer of transfer. Tumours induced by the agropine strain Bo542 synthesise agrocinopines C and D, phosphorylated sugar derivatives that differ from agrocinopines A and B in that they do not contain arabinose (Chapter 6). I tested whether agrocinopines C and D would induce transfer of agropine type Ti plasmids. A synthetic agropine analogue derived from the reduction of the Schiff base formed by the condensation of glutamic acid and mannose (Tempé *et al.*, 1980b) was used to select agropine Ti plasmid transconjugants.

This substance, referred to as 'mannopine' is a substrate for catabolic enzymes encoded by both octopine and agropine Ti plasmids (Tempé *et al.*, 1980). The agropine strains Bo542, NCPPB396 and NCPPB398 were induced to transfer their Ti plasmids by agrocinopine C. Transfer frequencies per donor were $3x10^{-3}$, $2x10^{-2}$ and $5x10^{-2}$ respectively. No transconjugants were observed when the donors had been precultured on 2.0mg/ml $(NH_4)_2SO_4$ plus 1mg/ml mannitol or on 2mg/ml mannopine and 2.0mg/ml $(NH_4)_2SO_4$. Preculture of a Bo542 on 3.8mM glucose equivalents of agrocinopine D as sole carbon source also induced the conjugative activity of pTi-Bo542. The other strains were not tested.

C. Does Agropine Induce Octopine Ti Plasmid Transfer?

Agropine (Firmin and Fenwick, 1978) occurs in tumours induced by octopine and agropine strains. Guyon *et al*. (1980) have shown that agropine is not an effective inducer of transfer of the agropine plasmid of Bo542 but no data has been presented on the effect of agropine on octopine Ti plasmid transfer. An experiment was carried out to determine whether agropine induces transfer of pTi-1001.

Tumours induced by a transconjugant strain C58Cl(pTi-1001) were extracted and the crude extracts subjected to electrophoresis as described in Chapter 6. Octopine was detected using the phenanthrene-quinone reagent (Yamada and Itano, 1966) and agropine was detected using alkaline silver nitrate reagent (Trevelyan *et al*., 1950); the results confirmed that this plasmid encodes genes that give rise to octopine and agropine biosynthesis.

For the conjugation experiment, the donor strain was NCPPB1001 and the recipient was C58ClRifStr. The donor was precultured on octopine or agropine (2mg/ml) as sole source of carbon and nitrogen. (Pure natural agropine was supplied by Dr M.E. Tate.) The donor grew on both substrates. The drop mating technique was used and transconjugants were selected on media containing octopine (2mg/ml) rifampycin (100μg/ml) and streptomycin (500μg/ml). Transconjugants arose when the donor had been grown on octopine medium but not agropine medium.

D. Does Octopine Induce Transfer of Octopine Ti Plasmids in Biovar 3 Strains

Two strains, K305 and K308, were used as donors, and crosses were carried out in the same experiment as described above in Section C. The octopine growth medium for the donors was supplemented with 0.1mg/ml yeast extract to supply growth factors required by the biovar 3 strains. No transconjugants arose, nor did they arise in replica plate crosses (Ellis, unpublished results; Kerr, personal communication).

Since the conjugative behaviour of these biovar 3 strains appeared different from biovar 1 octopine strains, the opine content of tumours induced by a representative of each biovar were compared to detect any differences in opine synthesis. The transconjugant strain C58Cl (pTi-1001) was the biovar 1 strain and K305 the biovar 3 strain. Agropine was readily detected in the tomato stem tumours induced by the biovar 1 strain but none was detected in the tumours induced by K305. The K305 tumours contained octopine.

DISCUSSION

Two important factors that must influence the maintenance and dispersal of a particular plasmid in a bacterial population are the selective advantage the plasmid confers on its host and the ability of the plasmid to spread by conjugation. With the Ti plasmids of *A. radiobacter*, opines are important for both these factors. Opines are the substrates of Ti plasmid encoded catabolic pathways and supply *A. radiobacter* with a nutrient supply that is not available to those

bacteria without the Ti plasmid. Some opines are also involved in the induction of conjugal transfer of the Ti plasmids. Octopine and its related pyruvic acid derivatives, octopinic acid and lysopine, induce transfer of octopine Ti plasmids (Petit *et al.*, 1978a; Tempe *et al.*, 1978; Klapwijk *et al.*, 1978). The process of induction is however, quite specific. For example, agrocinopine A but not nopaline induces transfer of nopaline Ti plasmids although both these opines occur in the same tumour tissue. Also agrocinopine C induces the transfer of agropine plasmids while agropine, a major opine product of agropine type tumours, does not induce conjugation (Guyon *et al.*, 1980).

The observation that nopaline does not induce transfer of nopaline Ti plasmids is in contradiction to some earlier reports (Kerr *et al.*, 1977; Petit *et al.*, 1978a; Hooykaas, 1979). How can these conflicting results be resolved? In those cases where nopaline has been reported to be an inducer of conjugation, transfer frequencies (expressed per recipient cell) have been low ($< 10^{-4}$) and mixtures of donor and recipient cells have been incubated for 24h or longer on media containing nopaline. Some of the previously reported data may be accounted for by selection and enrichment of rare transconjugants by nopaline in the conjugation medium, rather than induction of transfer functions.

Furthermore, some strains with nopaline Ti plasmids appear to be exceptional in their conjugative behaviour in that their Ti plasmids transfer without induction (Petit *et al.*, 1978b, and this Chapter).

One such strain, T37, was studied in more detail. Although the background level of transfer was quite high (10^{-5} per donor) the transfer frequency was increased to 10^{-1} by preinduction on agrocinopine A.

The two NOC plasmids (At-84b and pAt-112a) have an interesting conjugative behaviour. They are inducible by nopaline and by agrocinopine A. In this respect, they differ from all the nopaline Ti plasmids studied.

IIBV7 was the only nopaline strain that could not be induced to transfer its Ti plasmid. This strain is also insensitive to agrocin 84 (Kerr and Roberts, 1976). Murphy and Roberts (1979) have shown that sensitivity to agrocin 84 is due to a Ti plasmid linked uptake system. This permease is implicated in the uptake of agrocinopines A and B (Chapter 6). The failure of agrocinopine A to induce plasmid transfer of IIBV7 may therefore be due to the fact that this opine is unable to enter the bacterial cell. Alternatively, this strain may be transfer deficient (Tra^-). This possibility is supported by the fact that (an) important *tra* gene(s) lie(s) in close proximity to the agrocin sensitivity region of the nopaline Ti plasmids (Holsters *et al.*, 1980, Chapter 8).

The fact that nopaline does not induce Ti plasmid transfer and that Noc^c mutants of *A. radiobacter* are not constitutive for plasmid transfer is evidence that the regulation of nopaline catabolism and transfer are independent. However, Noc^c mutants of T37 have been reported which transfer the Ti plasmid constitutively (Kerr *et al.*, 1977; Ellis *et al.*, 1979a; Hooykaas, 1979). This is due to the

high uninduced level of transfer of pTi-T37 in the T37 background. The reason for this is unexplained. The plasmid pTi-T37Noc^{c} is not Tra^{c} in the C58Cl background (unpublished results).

Noc^{c} mutants of the avirulent strains K84 and K112 have been isolated. These mutants transfer constitutively (Ellis and Kerr, 1979; Ellis, unpublished results). This is consistent with the observations that nopaline induces transfer of both these non-oncogenic NOC plasmids.

An interesting pattern of induction of plasmid transfer by opines has emerged. For each Ti plasmid group, one group of opines whose synthesis they control is effective in inducing conjugation and the other ineffective. For example, agrocinopines A and B but not nopaline induce nopaline Ti plasmid transfer, agrocinopines C and D but not agropine induce agropine Ti plasmid transfer and octopine but not agropine induce octopine Ti plasmid transfer. This observation is discussed further in the final chapter of this thesis.

What is now known to be conjugal transfer of Ti plasmids was first observed by Kerr (1969, 1971) *in planta*, that is in crown gall tissue infected with a genetically marked Ti plasmid donor and a Ti plasmidless recipient. The results presented in this chapter and by earlier reports (Kerr *et al.*, 1977) show that *in planta* conjugative activity of Ti plasmids can be accounted for by induction of conjugation by specific opines in crown gall tissue. Guyon *et al.* (1980) have presented evidence for the generality of the opine concept of crown gall showing that the 'null type' or presumed opineless tumours induced by certain strains contain agropine which is a growth substrate for the inducing strain. This concept can now be extended. All Ti plasmids which have been observed to transfer *in planta* can be induced

to transfer *in vitro* by specific opines isolated from the galls induced by bacterial strains harbouring these plasmids. Since there is *in planta* conjugative transfer of the virulence plasmids in strains of *A. rhizogenes*, the pathogen responsible for 'hairy root' disease (Moore *et al.*, 1980; White and Nester, 1980) it is likely that, associated with hairy root disease, there is an opine that induces the transfer of these plasmids.

The octopine Ti plasmids in biovar 3 strains K305 and K308 were not induced by octopine to transfer to C58C1RifStr. Restriction of incoming plasmid DNA is not the explanation because transformants in the C58 background have been selected on octopine medium when plasmid DNA for transformation was isolated from biovar 3 strains K305 and K308 (Chilton *et al.*, in press). Further studies of the *in planta* and *in vitro* conjugal behaviour of these plasmids should be carried out to determine whether tumours induced by these strains contain a new opine which induces conjugation.

CHAPTER 8

ISOLATION AND PROPERTIES OF MUTANTS OF pTi-C58 THAT ARE CONSTITUTIVE FOR TRANSFER AND THEIR SIMILARITIES TO A Tra^{c} AGROPINE Ti PLASMID

INTRODUCTION

In the previous chapter, the role of agrocinopines A and B as inducers of transfer of the nopaline Ti plasmid, pTi-C58 was described. Without induction, the transfer of this plasmid occurs very infrequently ($< 10^{-7}$ transfers per donor). Holsters *et al.* (1980) however, isolated a mutant of pTi-C58 that transferred at high frequency without induction by opines. These authors proposed that this mutant plasmid was derepressed for transfer and designated the phenotype Tra^{c}. In this chapter, the isolation and properties of further independent Tra^{c} mutants of pTi-C58 are described. Conclusions drawn from these results are used to explain some curious properties of strain TC3, a transconjugant strain harbouring an agropine Ti plasmid (Guyon *et al.*, 1980).

EXPERIMENTAL AND RESULTS

A. ISOLATION OF Tra^{c} MUTANTS

Two donor strains were used, C58 and C58Arc^{+}D. The latter is a spontaneous mutant of C58 selected for the ability to grow on arginine medium. This strain is also able to grow on octopine medium. Arginine and octopine catabolism are selective markers for the Ti plasmid of this mutant (Chapter 3).

To isolate independent Tra^c mutants, the donor strain was patched onto a master plate of minimal medium and therefore was not induced to transfer by agrocinopines A or B. On the same day, the recipient strain C58ClRifStr was spread on selective medium containing rifampicin (100μg/ml) and streptomycin (500μg/ml) to counterselect the donor. When C58 was the donor, nopaline was used as the source of carbon and nitrogen to select Ti plasmid transconjugants; when $C58Arc^+D$ was the donor, octopine was used. After one day's growth, the donor patches were replica plated to the recipient lawn on selective medium and the plates were incubated for 10 days. Rare and isolated colonies that used the opines as substrate appeared on the thin background growth of the recipient lawn. These colonies must have arisen from independent transfer events and probably independent mutations of the donor plasmids to Tra^c, or alternatively from mutation of the donor to double antibiotic resistance. These colonies were purified and used as donors in a second round of conjugation with the recipient strain C58ClChlEry. Chloromycetin (100μg/ml) and erthromycin (100μg/ml) were used to counter-select the donors. If the donors were Tra^c, then they should retransfer the Ti plasmid at a high frequency. One Tra^c derivative of pTi-C58 was obtained. Out of 9 putative transconjugants selected from the cross $C58Arc^+D$ x C58ClRifStr, 7 transferred their Ti plasmids to C58ClChlEry. One Tra^c mutant of pTi-C58 and 7 Tra^c mutants of its Arc^+ derivative were obtained. A derivative of pGV3100, the Tra^c mutant obtained by Holsters *et al.* (1980), was also used in this study. This derivative, supplied as GV3804 from Ghent, Belgium, carries a Tn7 insertion in pGV3100. A Noc^c derivative of this plasmid was selected by plating GV3804 on octopine medium. I do not know the location of the insertion, but it has no detectable phenotypic effects on known Ti plasmid characters. The plasmid designations for these Tra^c mutants are listed in Table 8-1.

B. TRANSFER FREQUENCIES OF Tra^c PLASMIDS

The per donor transfer frequency of two Tra^c plasmids was determined as described in Chapter 7. The donors were pregrown on minimal medium and suspended in water before plating on strain C58ClRifStr growing on selective medium. In this experiment, pWI1000 transferred at 10^{-3} and pWI1003 at 2×10^{-2}. The transfer frequencies of the repressed plasmids pTi-C58 and pWI1001 were $< 10^{-7}$ per donor.

C. UPTAKE OF AGROCINOPINE A

Agrocinopine A can be used as sole source of carbon by strains harbouring nopaline Ti plasmids and also induces plasmid transfer. It was therefore of interest to study agrocinopine catabolism in a Tra^c mutant. The strain C58ClChlEry(pWI1000) was chosen for this study. The uptake of agrocinopine A by this strain was compared with wild type strain C58. Bacteria were grown overnight in liquid minimal medium to an OD_{680} of about 0.8. They were harvested and washed in the same medium without added ammonium sulphate or mannitol, resuspended and adjusted to an OD_{680} of 0.5. The suspension (10ml) was centrifuged and the bacteria resuspended in 1ml of the same salt solution and kept on ice. A 500µl aliquot of each bacterial suspension was transferred to a glass tube and 100µl of agrocinopine A solution added while the bacteria were cold. This gave a concentration of 2.5mM arabinose equivalents. A 60µl sample was taken immediately from both suspensions and spun in an Eppendorf bench-top centrifuge. At the same time the bacteria were placed in an orbital shaking water bath at 28^oC and further samples taken at 10 min intervals. After the samples were centrifuged, 50µl of the supernatant was transferred to a glass tube and kept on ice. When the sampling was completed, 1ml of phloroglucinol-HCl

Table 8-1.

PLASMID	ORIGIN
pTi-C58	wild type strain C58
pWI1000	pTi-C58 Tra^{c}, this study
pWI1001	pTi-C58 Arc^{+} from C58Arc^{+}D
pWI1002	Independent Tra^{c} derivatives
1003	of pWI1001
1004	"
1005	"
1006	"
1007	"
1008	"
pWI3804	derivative of pGV3100 with a Tn7 insertion (in strain GV3804)
pWI1009	pWI3804 Noc^{c} - this study . This plasmid enables its host to catabolise octopine and arginine

Figure 8-1. Uptake of agrocinopine A by C58 ● ● and C58ClChlEry(pWI1000) o o.

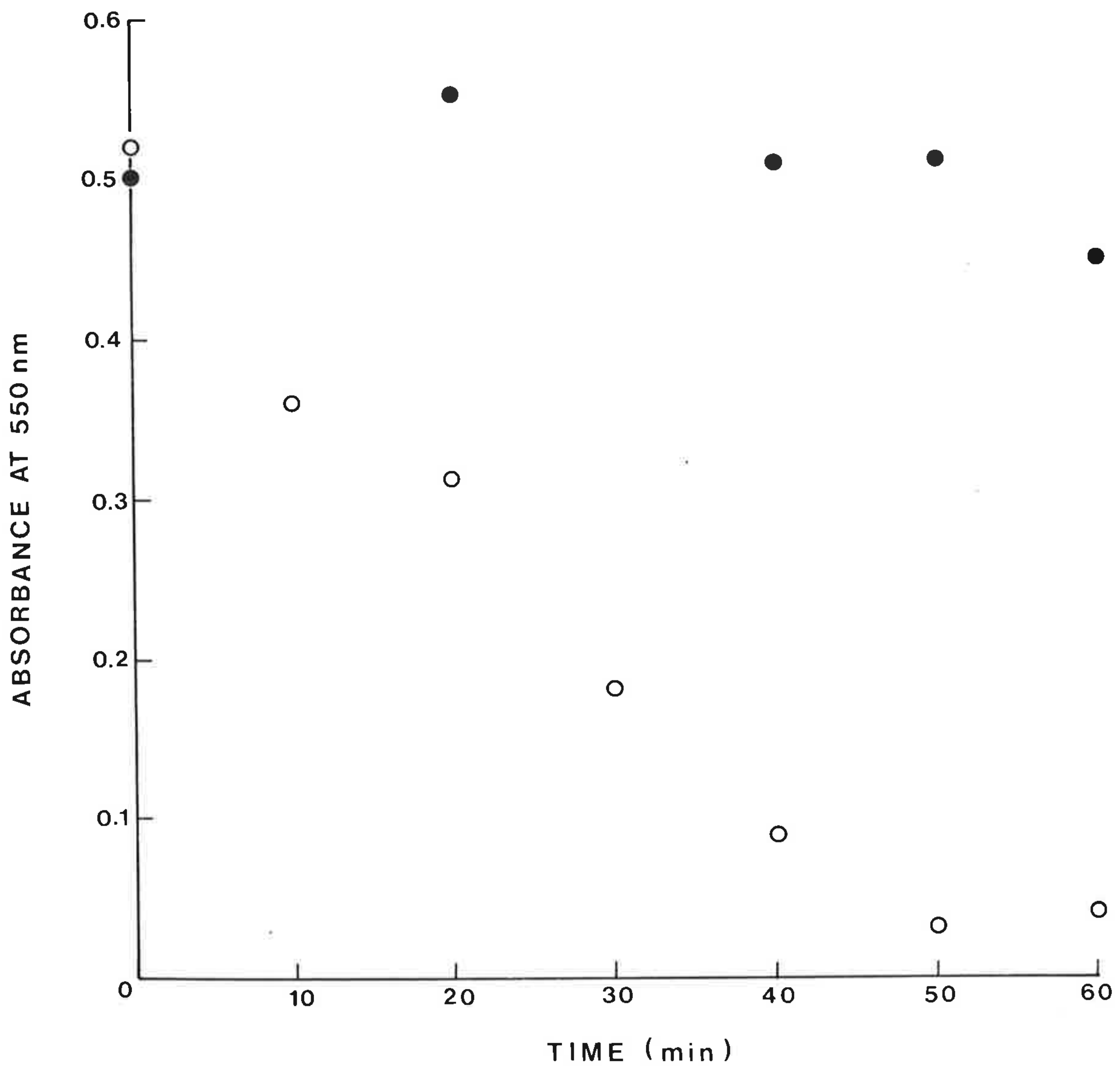
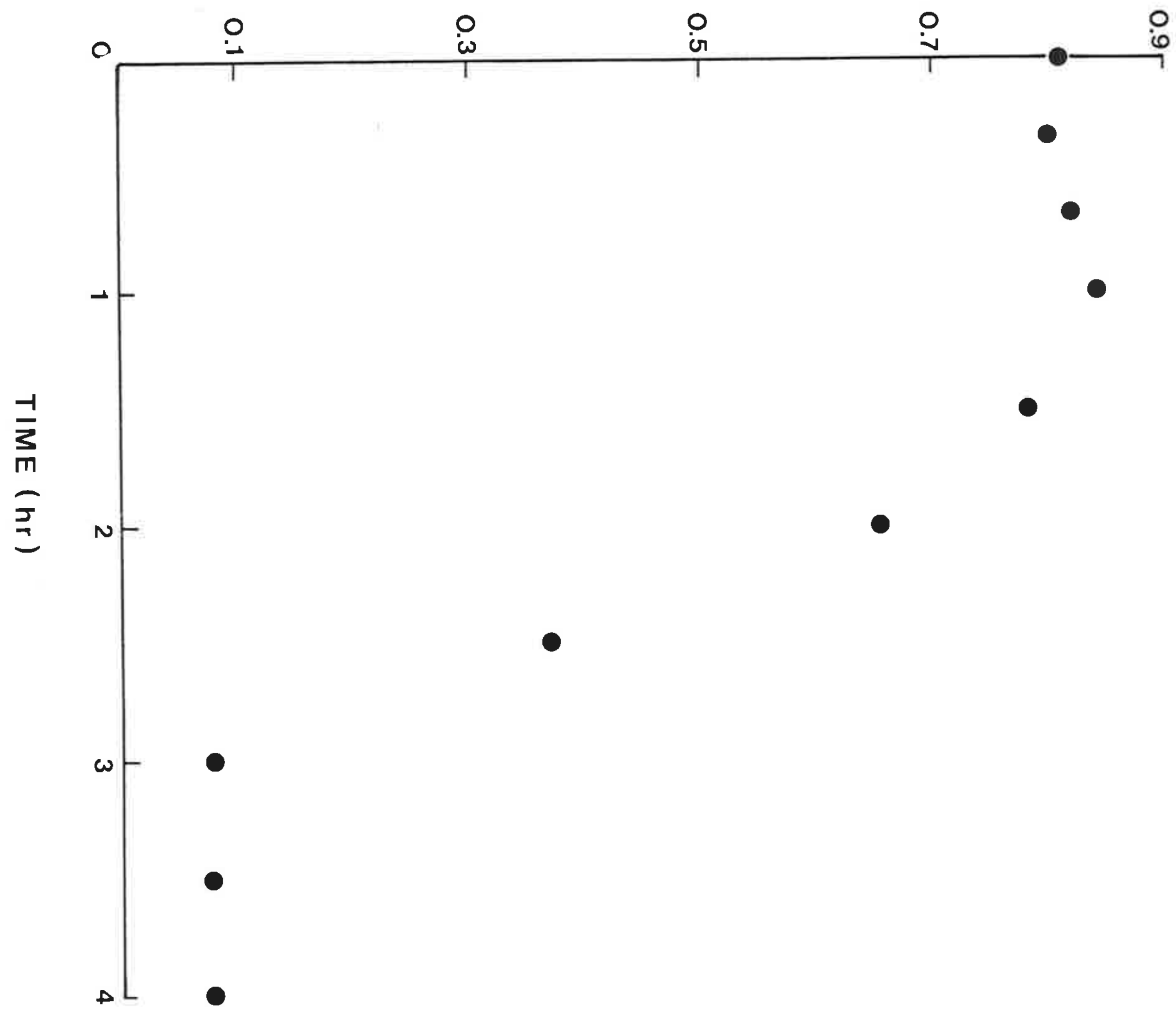

0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 550 nm
10
20
30
40
50
60
TIME (min)

Figure 8-2. Uptake of agrocinopine A by C58.


ABSORBANCE AT 550 nm
0
0.1
0.3
0.5
0.7
0.9
1
2
3
4
TIME (hr)

reagent (Chapter 6) was added to each tube which was sealed with glass marbles and boiled vigorously in a water bath for 10 min. The tubes were cooled in tapwater and the absorbance of the pink colour measured at 550nm. The results of one experiment are presented in Figure 8-1. The Tra^c mutant rapidly took up agrocinopine A. The wild type strain took up very little agrocinopine in the first hour.

In another experiment strain C58 was incubated in the presence of agrocinopine A and samples were taken over a 4-hour period. The result is presented in Figure 8-2. Strain C58 began to take up the agrocinopine after a lag of about 2 h. After this time, the uptake by C58 was as rapid as by the Tra^c strain in Figure 8-1. It appears then that the uptake system for agrocinopine A is inducible in strain C58 and constitutive in the Tra^c strain.

D. UPTAKE OF ^{32}P AGROCIN 84

Preculture of an agrocin 84 sensitive strain with agrocinopine A results in greatly increased uptake of [^{32}P] agrocin 84 and in induction of the transfer functions of nopaline Ti plasmids (Chapter 6 and 7). Therefore, the uptake of [^{32}P] agrocin 84 by two Tra^c mutants, C58Cl(pWI1000) and C58Cl(pWI1009) was studied. The experiments were carried out in collaboration with P.J. Murphy, as described by Murphy and Roberts (1979) (see Chapter 2 for details). The results for C58 and C58Cl(pWI1000) are shown in Figure 8-3. The results for C58Cl(pWI1009) were essentially identical as those for C58Cl(pWI1000). The Tra^c strain accumulated the maximum level of [^{32}P] agrocin 84 by 5 min. C58 did not reach this level until 40 minutes. From the

Fig 8-3

Uptake of [^{32}P] agrocin 84 (final concentration approximately 10^{-8}M and 60,000 cpm/ml) by C58 ●——● and C58ClChlEry(pWI1000) O——O.

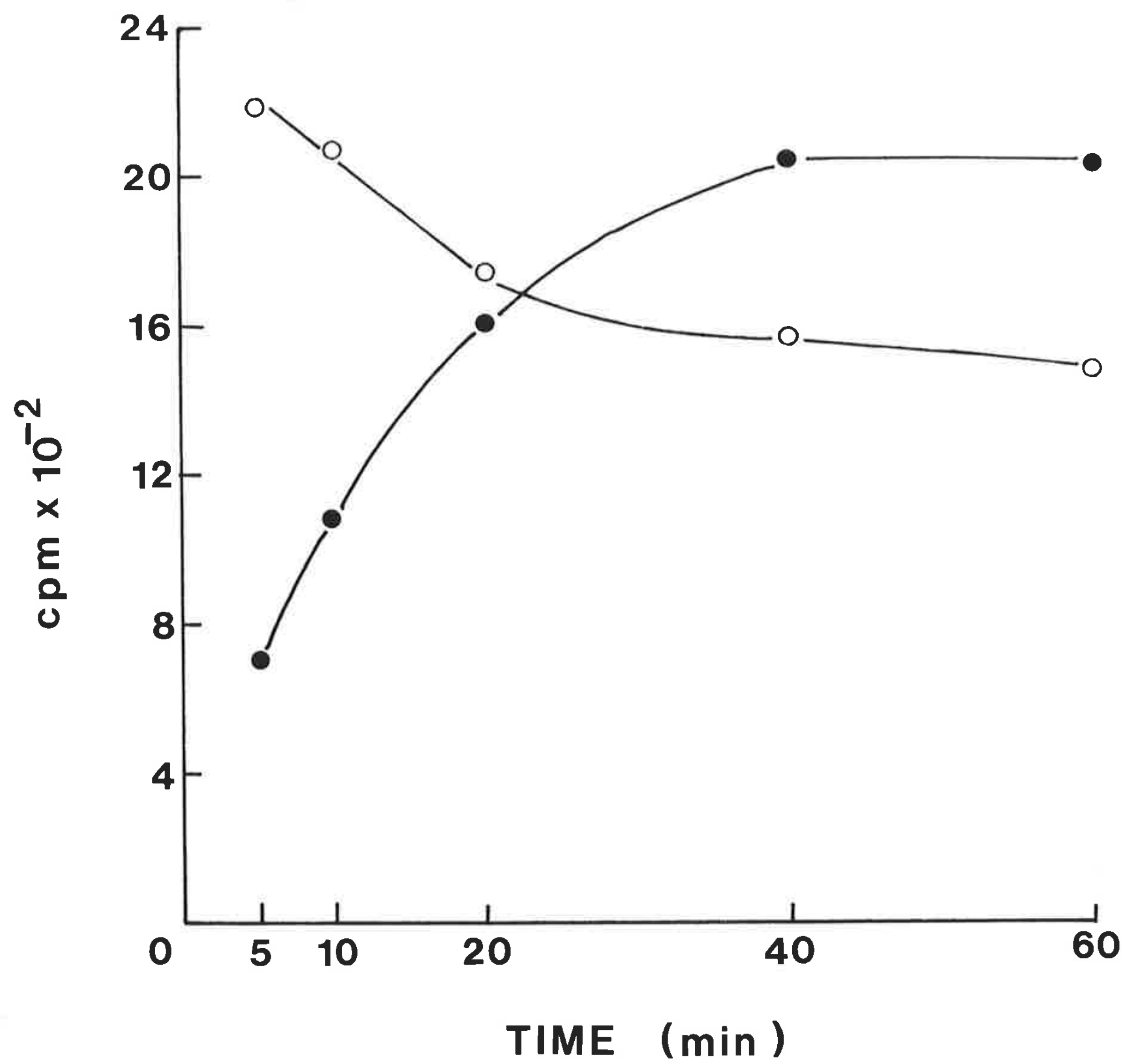
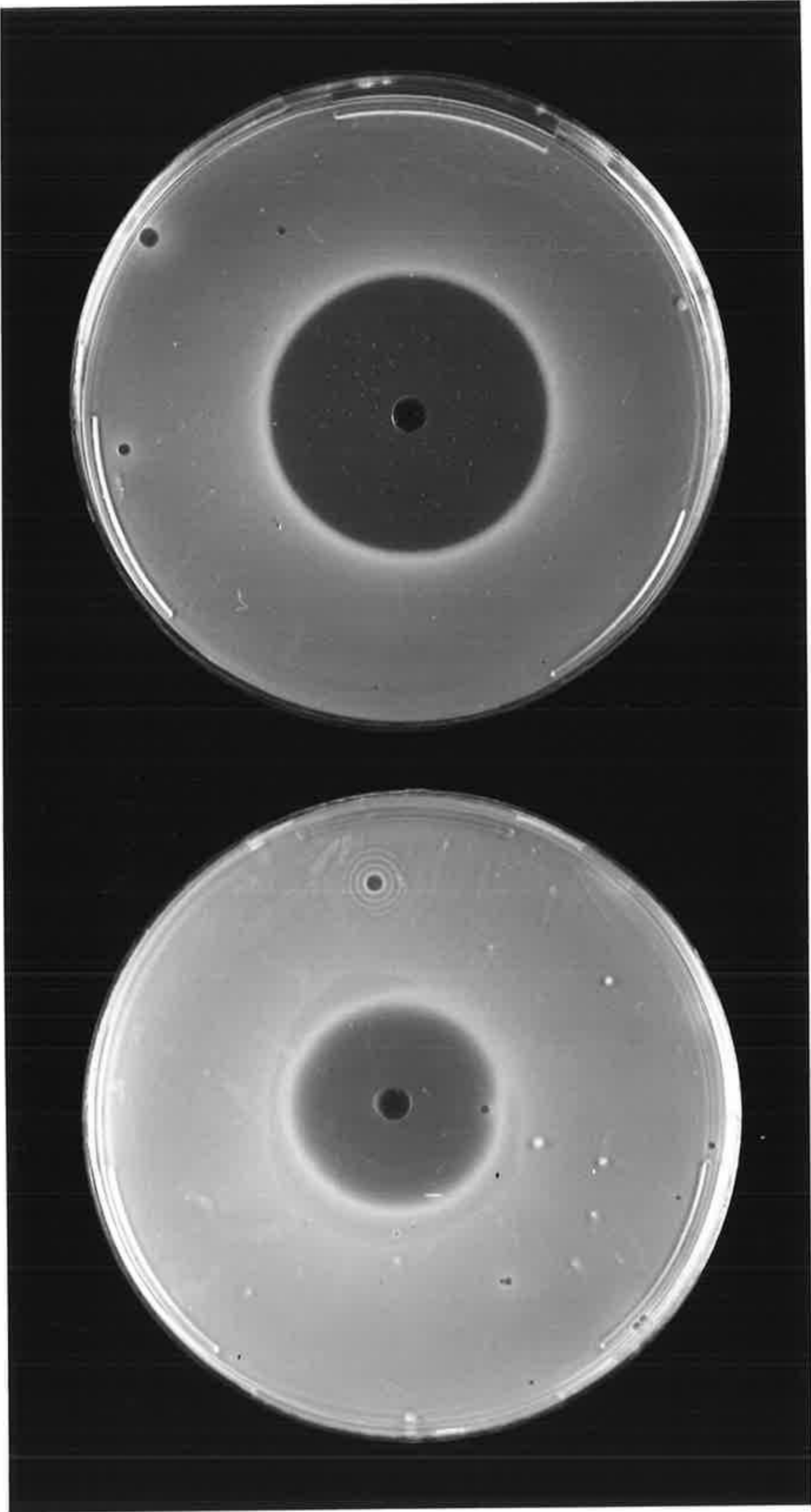

24
20
16
12
8
4
0
5
10
20
40
60
cpm x 10^{-2}
TIME (min)

Figure 8-4. Comparison of sensitivities of C58 and C58C1(pGV3102) to 20μl of agrocin 84 solution.

pGV3102 is a derivative of pTi-C58Tra^{c} (Holsters *et al.*, 1980).

C58 is the indicator strain in the top plate.



maximum level at 5 min, ^{32}P counts in the Tra^c strain fell. This could be due to loss of ^{32}P through the intact bacterial membrane or through cell lysis. The former is more likely since agrocin 84 does not cause cell lysis (Roberts, 1975).

The rapid uptake of [^{32}P] agrocin 84 by the Tra^c strains is correlated with supersensitivity of these strains to agrocin 84 in agar plate bioassays (see Chapter 2). When the Tra^c strains were used as indicators in the bioassay, very large zones of inhibition resulted. The experiment was repeated several times with the inclusion of strain C58 as a standard indicator. When the Tra^c strains were used as indicators for agrocin 84, the inhibition zones were markedly and consistently larger (from 1-1.5 cm in diameter) than the zones produced when C58 was the indicator. This effect is shown in Figure 8-4 where the sensitivities of C58 and a Tra^c strain to purified agrocin 84 are compared. The only exception to this observation was the strain harbouring pWI1003. This strain was resistant to agrocin 84. Whether this plasmid has undergone two separate mutations, one resulting in the Tra^c phenotype and the other in the Agr^r phenotype, or whether a single mutation has given rise to both phenotypes is not known.

E. THE EFFECT OF Tra^c Ti PLASMIDS ON AGROCIN 84 BIOSYNTHESIS

The loss of ^{32}P counts by C58Cl(pWI1000) and C58Cl(pWI1009) could be due to leakage of breakdown products of agrocin 84. Do Tra^c Ti plasmids break down agrocin 84? To investigate this all the Tra^c plasmids and also pTi-C58 were transferred to C58ClRifStr(pAt-84a). This strain harbours the bacteriocinogenic plasmid of strain 84 and

produces agrocin 84 (Chapter 5). The transconjungants were purified and tested for synthesis of agrocin 84 in the standard plate bioassay. Only transconjugants harbouring either pTi-C58 or pWI1003 produced agrocin 84. The other 8 plasmids 'turn off' agrocin 84 biosynthesis. How does this occur? One possiblity is that the Tra^{c} plasmids expel pAt-84a. This is not the case. The presence of pAt-84a in one transconjugant was confirmed by agarose gel electrophoresis (see Chapter 5, Figure 5-2, Lane C). This transconjugant, C58Cl(pWI1009)(pAt-84a) does not produce agrocin 84. However, when this strain was cured of its Ti plasmid by growth at 37°, the Ti plasmidless derivative C58Cl(pAt-84a) did produce agrocin 84, indicating that it is the presence of the Ti plasmid that 'turns off' agrocin 84 production. Since the wild type plasmid, pTi-C58 has no effect, the ability to 'turn off' agrocin 84 biosynthesis is associated with the Tra^{c} phenotype conferred by the mutant plasmids.

As was described in Chapter 5, FNT (a thermal degradation product of agrocin 84) inhibits strain C58Cl, but not C58Cl(pAt-84a). The bacteriocinogenic plasmid confers immunity on its host to this antibiotic. Transconjugant strains C58Cl(pWI1009) and C58Cl(pWI1109)(pAt-84a) were tested for their sensitivities to FNT. The former strain was sensitive and the latter immune. Therefore, although Tra^{c} mutants of pTi-C58 'turn off' agrocin 84 biosynthesis, they do not affect the expression of those functions of pAt-84a involved in immunity to FNT.

The only Tra^{c} plasmid that does not 'turn off' agrocin 84 synthesis is pWI1003. Neither does this plasmid encode sensitivity to agrocin 84. Are these two facts connected? This is considered in the next section.

F. PHENOTYPIC EFFECTS OF MUTATION TO AGROCIN 84 RESISTANCE

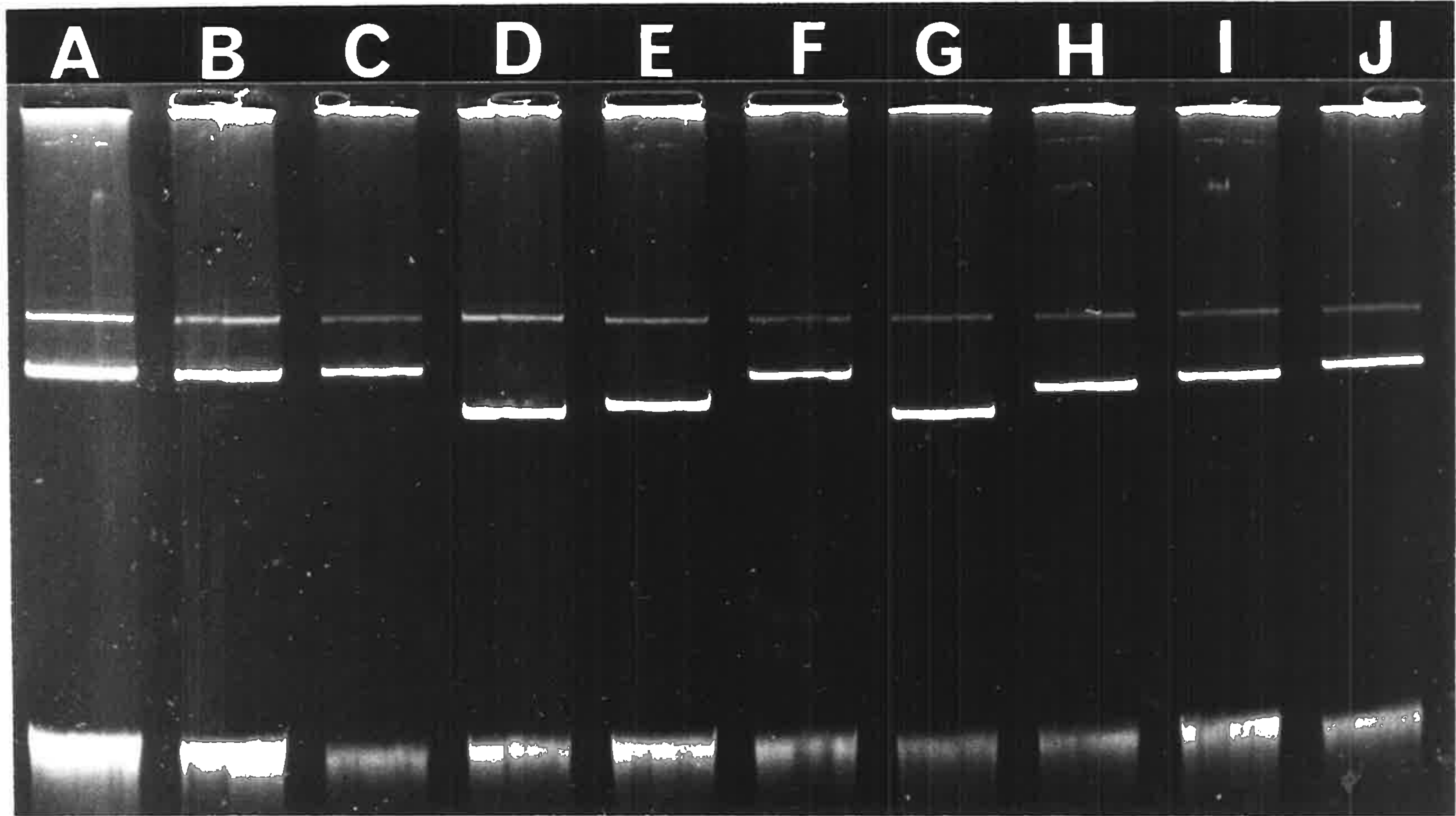
Agrocin 84 resistant mutants of strain C58C1ChlEry(pWI1009) were isolated by the following method. This strain was used as indicator in a standard plate assay for agrocin 84. The soft agar overlay within the inhibition zone was scraped off the plate and suspended in sterile water. The suspension was diluted and plated on arginine medium. Eighteen percent of the colonies grew rapidly on arginine, that is, they had the Arc^{+} phenotype. The Arc^{-} colonies were discarded because they had no selectable Ti plasmid trait.

The $Arc^{+}Agr^{r}$ mutants were purified through single colonies on arginine medium, tested for virulence and used as Ti plasmid donors to C58C1RifStr and to C58C1RifStr(pAt-84a). Out of 79 mutants, only 38 transferred their Ti plasmid. The remainder were Tra^{-}. The transconjugants of C58C1RifStr(pAt-84a) were tested for agrocin 84 production. All produced agrocin 84. Therefore, the ability of the parental $Tra^{c}Agr^{s}$ Ti plasmid to 'turn off' agrocin 84 production resides at or near the locus controlling sensitivity to the bacteriocin. Possible mechanisms to account for this effect are discussed at the end of this chapter.

All the $Agr^{r}Tra^{c}$ mutants were virulent on tomato seedlings. Most of the 41 $Agr^{r}Tra^{-}$ mutants were avirulent; only 5/41 were virulent. Three phenotypic classes of Agr^{r} mutants were isolated, namely $Tra^{c}Onc^{-}$, $Tra^{-}Onc^{-}$ and $Tra^{-}Onc^{+}$. No mutant was isolated that was $Tra^{c}Onc^{-}$. Plasmid profiles of examples of each of these phenotypic classes are shown in Figure 8-5. Several of these strains harbour Ti plasmids that have undergone large deletions. The fact that many of these

Figure 8-5. Plasmid DNA isolated from C58Cl(pWI1009) and its Agr^r mutant derivative electrophoresed in 0.7% agarose.

A = F = J	C58Cl(pWI1009)		Plasmid designation
B	Agr^r1	Tra^cOnc^+	pWI1010
C	Agr^r2	Tra^cOnc^+	pWI1011
D	Agr^r3	Tra^-Onc^-	pWI1012
E	Agr^r4	Tra^-Onc^-	pWI1013
G	Agr^r6	Tra^-Onc^-	pWI1014
H	Agr^r17	Tra^-Onc^+	pWI1015
I	Agr^r48	Tra^-Onc^+	pWI1016


A
B
C
D
E
F
G
H
I
J

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plasmids are Tra$^-$ and Onc$^-$ supports the genetic map of Holsters *et al.* (1980) who showed that a Tra region and Onc regions lie close to the Agr^S region of pTi-C58. It is curious that no Tra^{c}Onc$^-$ mutants were isolated. Perhaps proximal *tra* genes are located on either side of the Agr^S region; then any deletion extending from the *agr^S* gene into distal *onc* genes would also remove *tra* genes.

G. A STUDY OF STRAIN TC3

Strain TC3 is a transconjugant resulting from a cross between donor strain Bo542 and recipient C58C1RifStr and was selected on a medium containing agropine as source of nitrogen to select pTi-Bo542 (Guyon *et al.*, 1980). These authors reported that strain TC3 contains pTi-Bo542 and a 25 x 10^6 dalton cryptic plasmid derived from the donor. As described in Chapter 5, Bo542 produces a bacteriocin of the same specificity as agrocin 84. Is the small cryptic plasmid in TC3 the bacteriocinogenic plasmid from Bo542? This was investigated. TC3 was tested for agrocin production by the standard plate bioassay. It did not produce agrocin. However, it was immune to FNT. Strain A281, a transconjugant of NTI (Sciaky *et al.*, 1977) harbouring pTi-Bo542 was sensitive (NTI, like C58C1, is a Ti plasmidless-derivative of C58: Watson *et al.*, 1975). Therefore, the evidence indicated that the cryptic plasmid was a bacteriocinogenic plasmid and the transconjugant TC3 was analogous to the transconjugants described in Section E that contained pAt-84a and a Tra^c mutant of pTi-C58. TC3 was therefore tested for its ability to transfer pTi-Bo542 to C58C1ChlEry. Transconjugants were selected on mannopine medium (Chapter 7). TC3 indeed transferred pTi-Bo542 without induction with agrocinopines C or D. This strain therefore harbours a Tra^c

mutant of pTi-Bo542. This is consistent with the manner in which TC3 was originally isolated after conjugation of donor and recipient in the absence of agrocinopine C or D, the specific inducers of transfer of agropine Ti plasmids (Chapter 7).

In a later experiment, strain TC3 was used as a recipient for the NOC plasmid of strain 590Noc^c (see Chapter 5 for a description of this strain). Transconjugants were selected for their ability to catabolise octopine (the Noc^c donor phenotype) and the resistance of TC3 to rifampicin and streptomycin. Transconjugants were purified and were bacteriocinogenic, confirming that TC3 can, under the right circumstances, produce agrocin. The mechanism by which the NOC plasmid of 590Noc^c 'turns on' agrocin production has not been investigated further. It will possibly be explained in terms of incompatibility between the incoming NOC plasmid and the agropine Ti plasmid. The interaction between this NOC plasmid and the agropine Ti plasmid pTi-398 has been described in Chapter 5.

DISCUSSION

The one Tra^c strain examined in this chapter for uptake of agrocinopine A was constitutive for this character. Although the remaining mutants were not tested for agrocinopine A uptake, they (with the exception of pWI1003 which is Agr^r) shared with pWI1000 the correlated phenotypic effects of supersensitivity to agrocin 84 and the ability to 'turn off' agrocin 84 biosynthesis. From the data presented in this chapter and Chapter 7, a model in which a single repressor controls transfer and agrocinopine uptake functions is plausible. The comparison with the regulation of octopine uptake and

plasmid transfer by octopine Ti plasmids is obvious. Both octopine and nopaline Ti plasmids are induced by an opine substrate whose synthesis in tumour tissue they determine. Tra^c mutants of both plasmid types can be isolated which are constitutive for the functions involved in opine catabolism (Petit and Tempe, 1978; this chapter). Insufficient data are available at this stage to know whether the agrocinopine catabolic and transfer functions of nopaline Ti plasmids belong to separate operons, as do the octopine catabolic and transfer functions of octopine Ti plasmids (Petit *et al.*, 1978a; Petit and Tempé, 1978; Klapwijk *et al.*, 1978).

The basis of the Tra^c nopaline plasmids ability to 'turn off' agrocin 84 biosynthesis is unknown. The genes for this character reside in the region of the plasmid involved in determining agrocin 84 sensitivity and are under the same control as plasmid transfer functions. Two possibilities to explain the effect will be considered.

The Tra^c Ti plasmid encodes an increased level of agrocin 84 uptake. If a strain harbouring the bacteriocinogenic plasmid pAt-84a also harbours a Tra^c Ti plasmid, then any agrocin 84 produced that diffuses or is transported outside the cell will be rapidly taken up by the constitutive Ti plasmid-encoded permease. If uptake exceeds the movement of agrocin 84 out of the cell, then the cell will accumulate agrocin 84. This may cause feedback inhibition of agrocin 84 biosynthesis.

A second explanation is that the genes involved in catabolism of agrocinopines A and B may degrade agrocin 84 before it is released

from the cell. If this were the case, it is necessary to postulate that the breakdown product is still toxic since the Tra^c strains lacking the agrocin plasmid pAt-84a are sensitive to agrocin 84. If the product was non-toxic, then these strains should be resistant. The Ti plasmid would then be analogous to those R factors that encode enzymes that deactivate antibiotics. This is obviously not the case. If the product is toxic, why can't it be detected in bioassays? Several possibilities exist. The product may not be transported out of the producer cell. It may be of a lower toxicity than agrocin 84 or not taken up by indicator strains and therefore not detectable. The toxicity and specificity of agrocin 84 relies on the existence of a high affinity Ti plasmid encoded uptake system (Murphy and Roberts, 1979). The substitutions on the agrocin 84 molecule involved in uptake by sensitive strains may be absent from the degradation product.

The loss of ^{32}P counts by Tra^c strains that have accumulated [^{32}P] agrocin 84 supports the proposition that the Tra^c Ti plasmid is involved in breakdown of agrocin 84. Results presented in Chapter 6 support the idea that agrocin 84 and agrocinopines A and B are recognised and transported by the same permease. It is an intriguing possibility that the two molecules are also degraded by the same enzymatic system.

Preliminary evidence was presented that Tra^c agropine plasmids also 'turn off' agrocin production. This is not surprising in light of the results presented in this chapter and in Chapters 6 and 7. In Chapter 6 it was shown that the agropine plasmid pTi-Bo542 encodes agrocin sensitivity but this character is expressed only after induction

with agrocinopine C. A strain harbouring this plasmid also took up [^{32}P] agrocin 84 only after it was precultured in agrocinopine C. The transfer functions of pTi-Bo542 are also induced by agrocinopine C (Chapter 7). In these respects, the agropine Ti plasmid bears certain similarities to the nopaline Ti plasmids in the genetic control of transfer and their ability to express agrocin 84 sensitivity and agrocinopine transport and catabolic functions. Therefore the possible mechanisms by which the Tra^{c} nopaline Ti plasmid 'turns off' agrocin production are applicable to the Tra^{c} agropine plasmid.

Tempé *et al.* (1977) proposed that functions involved in the conjugal transfer of the Ti plasmid from bacterium to bacterium may also be involved in transfer of the Ti plasmid from bacterium to plant cell during tumour induction. The majority of the Tra^{-}Agr^{r} mutants isolated in this chapter were Onc^{-}. However, some were Onc^{+}. The mutants of the former phenotype can be explained in terms of deletions. Furthermore, Hernalsteens *et al.* (1978), Koekman *et al.* (1979) and Holsters *et al.* (1980) have isolated Tra^{-}Onc^{+} mutants. Ample evidence therefore exists to show that the two transfer processes are not identical. Nevertheless, insufficient data exist to dismiss this idea arbitrarily as done by Koekman *et al.* (1979). It is possible that certain functions are common to both processes and the close proximity of *tra* and *onc* genes on the Ti genes on the Ti plasmid is reason for further investigation.

CHAPTER 9

GENERAL DISCUSSION

One of the main aims of crown gall research has been to determine how *A. radiobacter* causes tumorous transformation of plant cells. This approach has been fruitful and has lead to the discovery of the Ti plasmids and their role as vectors for transfer of T-DNA to plant cells. However, an equally interesting question from the point of view of evolution that has received less consideration is why has *A. radiobacter* evolved such a system of pathogenicity? The ideas that have been formulated in the "opine concept" of crown gall throw light on this question. The production of opines has been described as "the driving force" in the evolution of crown gall (Tempé *et al.*, 1980a), and as the "biological rationale for the existence of the crown-gall tumor" (Guyon *et al.*, 1980). This situation, in which the parasite directs its host to supply a source of nutrients in a form available only to the pathogen is unique in all host-parasite relationships described so far. The fact that this is brought about by genetic engineering of a eukaryotic cell by a prokaryote makes the system more fascinating.

It is in the context of the opine concept that I wish to discuss the topic of biological control of crown gall. Although the work carried out at the Waite Institute on biological control through the production of agrocin 84 was recognized because of the effectiveness and economic importance of this control measure, it remained peripheral to the rapid advances made recently in basic research on the molecular biology of

crown gall disease. The connection between these two fields of research was made as early as 1974 when Roberts and Kerr (1974) demonstrated that virulence and sensitivity to agrocin 84 were closely correlated. It was later established that virulence and agrocin 84 sensitivity were linked and controlled by the nopaline Ti plasmid (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975; Engler *et al.*, 1975). However, this connection remained quite tenuous because there was no obvious function for a gene determining agrocin 84 sensitivity in the biology of crown gall.

The work of Murphy and Roberts (1979) and Tate *et al.* (1979), who demonstrated that the extraordinary selectivity of agrocin 84 is determined by a nopaline Ti plasmid-encoded high affinity uptake system which recognises particular structural features of the agrocin 84 molecule, provided some insight into the role of the gene(s) for agrocin 84 sensitivity. As it seemed unlikely that a "suicide gene" would have evolved in *A. radiobacter*, the idea of a non-toxic substrate for the uptake system was logical. The crown gall tumour seemed the most likely place to find such a substance. The discovery of the agrocinopines and their biological activity established a firm connection between the molecular biology and biological control of crown gall.

The results presented in Chapters 6 and 7 of this thesis show that the agrocinopines share the biological properties of the amino acid-derived opines such as octopine and nopaline. Biosynthesis of agrocinopines is determined by Ti plasmid gene(s) and the implication is that they are located in T-DNA. Furthermore, agrocinopine catabolism is also determined by Ti plasmid genes and it has been shown that agrocinopine A can serve as sole source of carbon for those strains harbouring a nopaline Ti plasmid.

The agrocinopines are also involved in promoting Ti plasmid transfer. These compounds must therefore play an important role in the ecology of nopaline and agropine Ti plasmids. The agrocinopines add further support to the opine concept since agrocinopines A and C sequester sucrose, the major transport sugar in plants.

The interaction of agrocinopines and agrocin 84 has some interesting implications which, although somewhat speculative, are worth discussing here. There is a clear implication that agrocin 84 and agrocinopines A and B share the same uptake system. Is this relationship fortuitous or is it a result of evolution? Strain 84 is a non-pathogenic organism but it is able to catabolise the tumour-specific opines, nopaline and agrocinopines A and B. In the environment of a nopaline crown gall tumour, strain 84 would be able to use these opines in competition with the pathogenic strains that had induced the galls. However, strain 84 has the advantage that it produces agrocin 84 which specifically inhibits these competing organisms. If such a system operates in soil and the success of biological control is supporting evidence that it does, then organisms such as strain 84 are well adapted to exploit the crown gall ecosystem at the expense of the pathogenic organisms. Under normal circumstances the situation could be envisaged where strain 84 colonises a tumour and kills the pathogenic strains. With biological control, this system has been manipulated so that strain 84 is introduced in large numbers to the plant roots before tumour induction has occurred.

Strain 84 has several other properties that may be important adaptions to its role in 'pirating' opines from tumours induced by other strains. In Chapter 8 I described how agrocin 84 biosynthesis did not occur when the bacteriocinogenic plasmid and a nopaline Ti plasmid

constitutive for transfer were harboured in the same cell. In contrast, when the NOC plasmid of strain 84 is constitutive for transfer, agrocin 84 synthesis is not affected. Evidence for this is in Chapter 5. Strain K84Noc^{c}Tra^c and its transconjugants synthesise agrocin 84. What are the implications of these observations? Consider a pathogenic strain that harbours both a wild type Ti plasmid and the agrocin 84 plasmid, in the environment of a crown gall tumour where opines are present. Agrocinopines A and B would induce the transfer functions of the Ti plasmid. If this derepressed plasmid behaved as the Tra^c mutants described in Chapter 8, then it would 'turn off' agrocin 84 production. Such a strain would have the advantage of the immunity to agrocin 84 conferred by the bacteriocinogenic plasmid but not the competitive advantage of agrocin 84 biosynthesis. In the same situation strain 84 would produce agrocin 84 since when the NOC plasmid in this strain is Tra^c, agrocin 84 biosynthesis is not affected. Unfortunately agrocinopines A and B were not available in sufficient quantity to test these predictions.

Why does a Tra^c nopaline Ti plasmid affect agrocin 84 biosynthesis yet a Tra^c NOC plasmid of strain 84 have no effect? The results presented in Chapter 8 show that when the Tra^c Ti plasmid is Agr^r, agrocin 84 synthesis is not affected. The NOC plasmid of strain 84 confers the Agr^r phenotype. When this plasmid was transferred to C58C1RifStr, the transconjugant was resistant to agrocin 84 (unpublished data). One piece of information is difficult to reconcile, however. This transconjugant, although Agr^r, is able to catabolise both agrocinopines A and B (unpublished data). The data presented in Chapter 6 indicate that the expression of agrocin 84 sensitivity is necessary for catabolism of agrocinopines. Agr^r mutants of Ti plasmids do not catabolise these

opines. There is no obvious explanation for the anomalous behaviour of the NOC plasmid of strain 84 unless the expression of agrocin 84 sensitivity requires both uptake of this molecule and an undescribed degradative step to yield a toxic product. It may be this step that strain 84 lacks.

One further difference between the nopaline Ti plasmids and the NOC plasmid of strain 84 involves the regulation of transfer. The Ti plasmids are induced by agrocinopines but the NOC plasmid is induced by both agrocinopines and by nopaline (Chapter 7). All the points of difference between this plasmid and the nopaline Ti plasmids indicate that the NOC plasmid of strain 84 cannot be considered as simply a nopaline Ti plasmid deficient in genes for virulence but rather as a plasmid that has evolved to suit its host as a colonizer of preformed nopaline galls.

In the light of the recent discoveries of first agropine (Firmin and Fenwick, 1978) and secondly the agrocinopines, I wish to redefine the three groups of Ti plasmids in terms of the opines they catabolise and the opines whose biosynthesis they control. Table 9-1 shows that each of the Ti plasmid groups, namely the octopine, nopaline and agropine Ti plasmids induce tumours that synthesise two distinct groups of opines. The octopine Ti plasmids induce tumours that produce what Guyon *et al.* (1980) refer to as pyruvic opines, which are pyruvic acid derivatives of the four basic amino acids arginine, ornithine, lysine and histopine. They also induce synthesis of agropine (Firmin and Fenwick, 1978), a mannose derivative (Tempé *et al.*, 1980; Tate, personal communication). The nopaline Ti plasmids, induce tumours containing α-ketoglutaric acid derivatives of arginine and ornithine (nopaline and nopalinic acid) and phosphorylated opines (agrocinopines A and B). The third group, the agropine Ti plasmids, induce tumours containing the mannose derivatives, agropine and phosphorylated opines (agrocinopines C and D).

Table 9-1. Ti plasmid types and the opines whose synthesis in crown gall tumours they control.

Plasmid class	Opines in tumour tissue			
	Pyruvic opines	α-ketoglutaric opines	Phosphorylated opines	Mannose opines
Octopine	octopine* octopinic acid* lysopine* histopine	–	–	agropine
Nopaline	–	nopaline nopalinic acid	agrocinopines A* and B*	–
Agropine	–	–	agrocinopines C* and D*	agropine

* Indicates opines that induce the transfer of the associated Ti plasmids.

In Chapter 6, I have described how a Nos$^-$ insertion mutant of pTi-T37 induces galls that synthesize agrocinopines A and B but not nopaline. This result implies that the synthesis of these two groups of opines is independent, which is not surprising when the chemical nature of these two groups is compared. Firmin and Fenwick (1978) have also proposed that different genes are involved in agropine and octopine biosynthesis. It is also most likely that different genes on the agropine Ti plasmids are involved in synthesis of agropine and agrocinopines C and D. If the two groups of opines are controlled by separate genes on each of the Ti plasmid groups, then T-DNA must carry at least three separate functional regions; one region involved in oncogenicity and two involved in opine synthesis.

It is interesting to note that only one of the two groups of opines induces transfer of each Ti plasmid type. Only the pyruvic opines, with the exception of histopine, induce transfer of octopine Ti plasmids (Petit *et al.*, 1978a). Agropine does not induce transfer of the octopine plasmid pTi1001 (Chapter 7). Agrocinopines A and B, but not nopaline, induce nopaline Ti plasmid transfer (Chapter 7) and agrocinopines C and D, but not agropine, induce agropine Ti plasmid transfer (Chapter 7; Guyon *et al.*, 1980).

Some of these opines have been quantified in a range of crown gall tissues by Scótt *et al.* (1980). In octopine tumours the levels of agropine are much higher than the levels of pyruvic opines, yet it is the pyruvic opines that induce conjugal transfer of octopine Ti plasmids. Although the opines in agropine tumours have not been quantified, agropine levels

appear equivalent to those in octopine galls and much higher than levels of agrocinopine C when detected on paper electrophoretograms by alkaline silver nitrate reagent (Ellis and Tate, unpublished results). Again, it is the minor opines, agrocinopine C and D which induce conjugation. In the case of nopaline tumours, nopaline is at much higher levels than the pyruvic opines in octopine tumours and in general, nopaline occurs at equivalent levels to agropine in octopine tumours (Scott *et al.*, 1980). Although agrocinopine A and B have not been quantified, it seems that nopaline and nopalinic acid (α-ketoglutaric opines) are the major opines in nopaline tumours (Ellis, unpublished results), and these opines are not active in inducing conjugation. The pattern that emerges is that crown gall tumours contain a major opine group whose function is mainly nutritional, and a minor group of opines which are inducers of plasmid transfer.

To conclude this chapter I wish to discuss some of the gaps remaining in the understanding of crown gall. First there is the question of how the T-DNA enters the plant cell. Does the total Ti plasmid enter to be processed down to the T-DNA? There is evidence (Lippincott and Lippincott, 1969) to indicate that adherence of the bacterium to the plant cell may be a requirement for infection. What follows is unknown.

The process by which T-DNA induces tumorous transformation of plant cells has not been elucidated. Schell and Van Montagu (1978) proposed that T-DNA may be a transposon. Insertion of T-DNA in the plant genome may cause mutation of a function involved in control of cell division and differentiation. The transposon model in this simple form is probably not correct because T-DNA maintained in various octopine tumour

lines is of variable length (Thomashaw *et al.*, 1980) and at least the right-hand boundary of T-DNA varies between tumour lines (Merlo *et al.*, 1980). The T-DNA maintained in tumour DNA therefore does not have constant flanking sequences as do bacterial transposons (Cohen, 1976).

An interesting model for the role of T-DNA in tumour induction can be drawn directly from a consideration of opine synthesis. Opine synthesis can be considered in two ways. First, it can be considered as the synthesis of novel plant metabolites not present in normal plants. Does the T-DNA encode an undiscovered novel metabolite which is an inducer of cell division? Secondly, opine synthesis can be considered as the removal of certain normal plant metabolites from the cellular pool by their conversion to a different form. For example, nopaline synthesis may be considered as the removal of arginine from the intra-cellular pool. Does the T-DNA encode enzymes which remove important control molecules involved in regulation of cell division?

Another proposal to explain tumour induction by insertion of T-DNA has been put forward by Gurley *et al.* (1979). They proposed that the T-DNA may provide an internal promoter that enables read-through transcription of genes that are not expressed by differentiated or non-dividing cells. This idea is interesting but the results of Yang *et al.* (1980) and McPherson *et al.* (1980) imply that a T-DNA product may be involved in tumour induction. Yang *et al.* (1980) have shown that transcription occurs throughout the T-DNA region in tumour cells and McPherson *et al.* (1980) have shown that messenger RNA that hybridizes with the common region of the T-DNA can be translated to polypeptide

products *in vitro* and proposed that this polypeptide may be involved in oncogenicity. The elucidation of the mechanism of transformation should have interesting implications on the control mechanisms of plant cell division and differentiation.

APPENDIX I

PUBLICATIONS

Ellis, J.G. and Kerr, A. Developing biological control agents for soilborne pathogens. Proc. 4th Int. Conf. Plant Path. Bact., Angers 1978, pp. 245-256.

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Agrobacterium: genetic studies on agrocin 84 production and the biological control of crown gall

J. G. Ellis, A. Kerr,

Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064

M. van Montagu and J. Schell

State University, Gent, Belgium

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J. G. Ellis, A. Kerr,

Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064

M. van Montagu and J. Schell

State University, Gent, Belgium

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The rôle of the bacteriocin, agrocin 84, in the biological control of crown gall was investigated by constructing new bacteriocinogenic strains. A small plasmid which codes for the ability to produce agrocin 84 has been identified and transferred to recipient strains by conjugation. Two donor strains were used; the avirulent strain 84 and the virulent strain 398. Agrocin production and virulence in the latter strain have been separated by genetic manipulation and avirulent, agrocin producing derivatives have been produced.

Avirulent transconjugants which produced agrocin 84 varied in their effectiveness as biological control agents. Evidence is presented that this was probably due to variation in rate of growth of the different strains in the inoculation sites.

INTRODUCTION

A remarkably successful biological control method has been developed in this laboratory that prevents crown gall induction on stone fruit trees in southern Australia [*11*] and in other parts of the world [*5*, *18*, *24*]. Susceptible planting material is dipped in a cell suspension of avirulent strain 84 of *Agrobacterium radiobacter* var. *radiobacter*. This strain produces a highly specific nucleoside bacteriocin called agrocin 84 [*23*]. Kerr & Htay [*13*] proposed that biological control is due to the production of this bacteriocin since only those strains of *Agrobacterium* sensitive to agrocin 84 are subject to biological control.

Virulent agrobacteria sensitive to agrocin 84 harbour the nopaline type Ti plasmid which determines virulence [*27*, *29*], nopaline catabolism [*2*] and sensitivity to agrocin 84 [*7*]. Some strains however are resistant to agrocin 84 and are not subject to biological control, e.g. the biotype 3 strains which are of considerable economic importance in causing crown gall of grapevine [*14*].

With the aim of extending biological control to *Agrobacterium* strains resistant to agrocin 84, Kerr & Panagopoulos [*14*] attempted to isolate new bacteriocinogenic strains from soil. These strains and several new strains that produce bacteriocins of different specificities have proved ineffective as biological control agents (our unpublished results). So, in an effort to understand why K84 has proved so effective in contrast to these new bacteriocinogenic strains, we have looked more closely at strain K84 and its bacteriocin.

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If biological control of crown gall is due to agrocin 84 production, it should be possible to construct new biological control agents by transferring this character to other organisms. This was attempted. We show that agrocin 84 production in strain K84 is controlled by a transmissible plasmid of 30×10^6 daltons; by transferring this plasmid to various avirulent recipients, bacteriocinogenic transconjugants, which we have tested as biological control agents, have been produced. The rôle of agrocin 84 in biological control is discussed.

Another strain, NCPPB 398, also produces an antibiotic which appears identical to agrocin 84 (W. P. Roberts, pers. comm.). It is a biotype 1 pathogen which harbours a "null" type Ti plasmid. This plasmid does not code for the ability to catabolize either octopine or nopaline and induces tumours that synthesize neither opine [*15*]. We show that agrocin production in this strain is also controlled by a transmissible plasmid slightly smaller than the agrocin plasmid in strain K84. Avirulent strains harbouring the 398 agrocin plasmid have been constructed and tested as biological control agents.

MATERIALS AND METHODS

Bacterial strains and typing bacteriophages

The bacterial strains are listed in Table 1. Biotype designations are according to Keane *et al.* [*12*] and Kerr & Panagopoulos [*14*]. The typing set of bacteriophages has been described [*8*].

TABLE 1

Bacterial cultures used

Strain	Biotype	Virulence	Origin
C58-C1†	1	—	C58 cured of its Ti plasmid: J. Schell, Gent
C58-C1RP4	1	—	C58C1 containing drug resistance plasmid RP4: J. Schell, Gent
θ5†	1	—	B91 cured of its Ti plasmid: J. Schell, Gent
398†	1	+	NCPPB
590	1	—	A. G. Lockhead
57 (pTi27)	1	+	Kerr and Roberts [*15*]
27	2	+	Peach gall, S. Australia
K84	2	—	Soil around peach gall, S. Australia
108	2	+	Soil around almond gall, S. Australia

† Antibiotic resistant mutants of these strains were also used and are designated by the suffices Rif (resistant to 25 $\mu g\ ml^{-1}$ rifampicin), Str (resistant to 500 $\mu g\ ml^{-1}$ streptomycin) and Chl (resistant to 125 $\mu g\ ml^{-1}$ chloromycetin).

Media

Minimal medium contained, per litre: K_2HPO_4, 10·5 g; KH_2PO_4, 4·5 g; $MgSO_4.7H_2O$, 0·2 g; $CaCl_2$, 10 mg; $FeSO_4$, 5 mg; $MnCl_2$, 2 mg and agar, separately sterilized, 20 g.

Conjugation medium consisted of minimal medium plus 200 mg per litre of separately sterilized octopine, kindly supplied by Monsieur J. Tempé, I.N.R.A., Versailles.

Selective medium consisted of minimal plus, per litre, 2 g octopine as sole carbon and nitrogen source, or 200 mg octopine as nitrogen source and 1 g glucose as carbon source. Streptomycin, 0·5 g, and rifampicin, 25 mg, were used to counterselect donors. (In one experiment 125 mg chloromycetin was used.) Octopine, glucose and antibiotics were sterilized separately and added to the medium just before plates were poured.

Yeast mannitol (YM) medium contained, per litre: K_2HPO_4, 0·5 g; $MgSO_47H_2O$, 0·2 g; NaCl, 0·2 g; $CaCl_2$, 0·2 g; $FeCl_3$, 0·01 g; yeast extract, 1·0 g; mannitol, 10 g and agar 15 g.

PA medium contained per litre, peptone 4 g, $MgSO_2$ 2 ml of a 1 M stock solution.

Selection of mutants constitutive for nopaline catabolism (Noc^c)

Octopine is a non-inducing substrate for the nopaline catabolic system of *Agrobacterium* and Noc^c mutants of nopaline strains can catabolize octopine [*21*, *22*]. To select Noc^c mutants, approximately 5×10^8 cells were plated on to minimal medium containing, per litre, either 200 mg octopine and 1 g glucose or 2 g octopine only. Noc^c mutant colonies appeared after 10–14 days' incubation at 27 °C.

Transfer of nopaline catabolic plasmids and agrocin production plasmids

Noc^c donor strains were applied as patches on to a master plate of conjugation medium. Approximately 5×10^8 recipient cells, resistant to antibiotic(s), were spread on a separate plate of conjugation medium. Both plates were incubated overnight at 27 °C and then the donors were replica plated on to the recipients. After 24 h, this plate was replica plated on to selective medium. Patches of octopine-utilizing cells appeared after 4 to 8 days and were purified by streaking on selective medium. Transconjugants were tested for agrocin production on the medium of Stonier [*26*] using the replica plating method described by Kerr & Panagopoulos [*14*].

Plasmid isolation and electrophoresis

The plasmid isolation procedure was based on the Currier & Nester method [*4*] except that all precautions were taken to avoid shearing of the DNA. All solutions (except protease) and glassware were autoclaved to eliminate nuclease activity.

Bacteria were grown in 25 ml of PA medium to an O.D.$_{680}$ of about 0·3. Cells were collected and washed once in 5 ml of TE8 buffer [*10*], then resuspended in 4 ml of the same buffer. Cells were lysed in a 25 ml tube by adding 0·5 ml of protease solution (Sigma Type VI, 5 mg ml^{-1} in TE8, self-digested at 37 °C for 60 min) and 0·5 ml of SDS solution (100 mg ml^{-1} stock solution in TE8). Clear lysates resulted after 30 to 40 min at 37 °C. DNA was denatured by raising the pH to 12·1 to 12·3 with about 180 μl of 2 N NaOH. After 10 to 15 min gentle inversion, the DNA was returned to pH 8·5 to 9·0 with approximately 350 μl of 2M Tris–HCl pH 7. The DNA was gently agitated with a glass rod until all viscosity disappeared and then adjusted to 3% NaCl by adding 0·6 ml of 5 M NaCl, mixed by a single inversion, then extracted with an equal volume of redistilled phenol equilibrated with 3% NaCl. After several rapid inversions to mix the phases, the contents of the tubes were mixed by gentle inversion for 7 min. The aqueous phase was separated by centrifugation and removed using the wide end of a 5 ml pipette to avoid shearing. The aqueous phase was

adjusted to approximately 0·3 M Na acetate (0·75 ml of a 3 M stock solution) and approximately 1 M NaCl (1·5 ml of a 5 M stock solution). DNA was precipitated at −20 °C overnight with two volumes of absolute ethanol. The precipitate was collected by low speed centrifugation (2500 *g*) for 10 min then redissolved in 120 μl TES 8 [*10*] and 40 μl of tracking dye (20% ficoll, 0·1% SDS, 0·02% bromophenol blue). Samples of 20 to 40 μl were electrophoresced in 0·7% agarose essentially as described by Meyers *et al.* [*17*]. Horizontal slab gels were used and electrophoresis was for 3 h at 150 V (about 1 V cm^{-1} length of gel). DNA was stained with 0·5 μg ml^{-1} ethidium bromide solution in Tris–borate running buffer for 45 min. Gels were irradiated with short wavelength u.v. and photographed with Ilford Pan F film using a yellow gelatin filter.

RESULTS

Genetic transfer of agrocin production

Donor strain K84. The 124×10^6 dalton plasmid in K84 [*16*] which codes for the ability to catabolize nopaline [*25*] was used as a conjugative plasmid in an effort to transfer the ability to produce agrocin 84 to various recipient strains. The transfer functions of this plasmid are normally repressed and require induction by nopaline [*6*]. Since nopaline is not readily available we carried out most crosses using a mutant of K84 which is constitutive both for nopaline catabolism and for transfer. This mutant is designated K84 Noc^c and is able to grow on octopine as the sole source of nitrogen or of carbon and nitrogen.

Initial experiments in which a wild type or a constitutive nopaline catabolic plasmid was transferred to C58C1RifStr confirmed the results of the transformation experiments of Sciaky *et al.* [*25*]; the ability to produce agrocin is not a marker for this plasmid. Furthermore, this plasmid does not code for agrocin 84 sensitivity, a character associated with most nopaline Ti plasmids [*7*, 15].

Since agrocin production was not linked to the nopaline catabolic plasmid of strain K84, we decided to test a large number of nopaline catabolizing transconjugants for possible co-transfer of agrocin 84 production. In one experiment, using K84 Noc^c as donor, we found that about 10% of the transconjugants did indeed produce agrocin 84. Similarly the ability to produce agrocin 84 was transferred to θ5RifStr. The phenotypes of the donor, recipients and transconjugants are given in Table 2.

Donor strain NCPPB398. We attempted to transfer agrocin production from strain 398 to C58C1. To establish a conjugation system in strain 398, we introduced a selectable conjugative plasmid. A plasmid, which codes for constitutive nopaline (and consequently octopine) catabolism, agrocin 84 sensitivity but not for virulence, was introduced by conjugation to strain 398RifStr from donor strain $590Noc^c$. Since strain 398 does not grow on octopine, transconjugants could be selected for their ability to grow on octopine. Ten independent transconjugants were selected; nine were virulent and one was avirulent. The reason for loss of virulence is under investigation. This avirulent transconjugant which still produces agrocin will be discussed further as a potential biological control agent.

Having introduced a selectable conjugative plasmid to strain 398RifStr, we were able to use this transconjugant as a donor in a second round of conjugation. We used

TABLE 2

Characteristics of donor strain 84Noc^c, recipient strains (C58-C1RifStr and θ5RifStr) and trans-conjugants

	Octopine utilization	Agrocin 84 production	3-Ketolactose† production	Phage pattern S1	S2	S3	S5	S6
84Noc^c	+	+	−	−	−	−	−	−
C58-C1RifStr	−	−	+	−	+	−	+	+
θ5RifStr	−	−	+	+	−	+	−	−
Transconjugants								
84Noc^c × C58-C1RifStr# 1	+	−	+	−	+	−	+	+
#2	+	+	+	−	+	−	+	+
84Noc^c × θ5RifStr# 1	+	−	+	+	−	+	−	−
#2	+	+	+	+	−	+	−	−

† This test is described by Bernaerts & Deley [1] and distinguished biotype 1 and 2 agro-bacteria [12].
+ = sensitive; − = resistant.

the virulent transconjugant 590Noc^c × 398RifStr# 5 as donor and C58C1Chl as recipient. Again transconjugants were selected on octopine as the sole source of carbon and nitrogen; 125 μg ml^{-1} chloromycetin was used to counter select the donor. Transconjugants were tested for (1) agrocin production, (2) agrocin sensitivity and (3) virulence; four classes of transconjugant appeared (see Table 3). In some cases agrocin production was transferred from the 398 genetic background to C58C1Chl showing that the agrocin production trait in 398 is also transmissible. The virulence trait of the null type Ti plasmid was co-transferred to 16/75 transconjugants.

Plasmid involvement in agrocin 84 production and immunity

It seemed plausible that such a high frequency of co-transfer of the ability to produce agrocin 84 was due to mobilization of a plasmid coding for agrocin production. We screened our bacteriocinogenic donor strains, the recipients and transconjugants for plasmid DNA (Plate 1).

The ability of the transconjugants to produce agrocin 84 was always associated with the presence of a small plasmid which had been co-transferred with the nopaline catabolic plasmid from the donors. The same results were obtained with the θ5 transconjugants (results not shown). Merlo & Nester [16] showed that the small plasmid of K84 is about 30 × 10^6 daltons. From its relative mobility (see Plate 1) the agrocin plasmid of strain 398 appears slightly smaller than the K84 plasmid.

In addition to the agrocin plasmids, other plasmids are present including very large cryptic plasmids which occur in most strains of *Agrobacterium*. The open circular form of the cryptic plasmid from C58C1 has been measured as 280 × 10^6 daltons (our unpublished results).

It appears that the agrocin plasmids also code for immunity to agrocin 84. The evidence for this appears partly in Table 3. The agrocin 84 sensitivity coded for by the 590Noc^c nopaline catabolic plasmid is not expressed in the bacteriocinogenic transconjugants of the 590Noc^c × 398RifStr cross. However, when the Noc^c plasmid was retransferred to C58C1Chl in the second round of conjugation, agrocin sensitivity reappeared in some transconjugants. In all cases, sensitivity was expressed only in

TABLE 3

Characteristics of the strains involved in crosses with strain 398RifStr or its derivatives

	Octopine utilization	Agrocin 84 production	Agrocin 84 sensitivity†	Virulence
590Noc^c	+	−	s	−
398RifStr	−	+	r	+
590Noc^c × 398RifStr# 1	+	+	r	−
# 5	+	+	r	+
C58-C1Ch1	−	−	−	−
(590Noc^c × 398RifStr# 5) × C58-C1Ch1				
Transconjugant class A	+	+	r	+
B	+	−	s	−
C	+	−	s	+
D	+	+	r	−

† s = sensitive; − = not sensitive; r = resistant.

non-bacteriocinogenic transconjugants. Bacteriocinogenic transconjugants were immune. Furthermore, Ellis & Kerr [*6*] and Panagopoulos *et al.* [*20*] showed that when the ability to produce agrocin 84 was transferred from K84 to agrocin 84 sensitive pathogens, the transconjugants became immune to agrocin 84, yet lost no other Ti-linked character.

Bacteriocinogenic transconjugants as agents of biological control

From the bacterial crosses described in the previous section, several avirulent strains producing agrocin 84 were obtained. Most were tested for the control of crown gall induction on tomato stems [*13*]. In one experiment, all strains listed in Table 2 were tested against the virulent biotype 1, agrocin 84 sensitive strain 57 (pTi27). Only the donor strain 84Noc^c and transconjugant no. 2 from the cross 84Noc^c × C58-C1RifStr prevented crown gall induction, indicating that the ability to achieve biological control is transferred with agrocin 84 production. Surprisingly, the bacteriocinogenic no. 2 from the cross 84Noc^c × θ5RifStr was not effective in biological control (Plate 2).

It is clear from these results that agrocin 84 production is not the only character required to control crown gall. We tested the hypothesis that the failure of one bacteriocinogenic strain to control crown gall induction was due to poor growth of the organism in the inoculation sites. Stems of 4-week-old tomato plants were inoculated either with strain 27 which is virulent, biotype 2 and agrocin 84 sensitive, or with the same strain mixed in a ratio of 1 : 1 with one or the other of the two agrocin-producing transconjugants. There were 10 replicates of each treatment. Immediately after inoculation and daily for the next 4 days, inoculated stem segments of two plants from each treatment were macerated in water, diluted and plated on selective media—the medium of New & Kerr [*19*] for strain 27 and yeast mannitol agar supplemented with 500 μg ml^{-1} streptomycin, 25 μg ml^{-1} rifampicin and 250 μg ml^{-1} cycloheximide for the biotype 1 transconjugants. Duplicate plates were used for each plant. Results are given in Fig. 1. The ineffective strain did not multiply rapidly in the wounds and inhibited strain 27 much less than did the effective strain. In fact, the only inhibition detected was at time 0, and perhaps

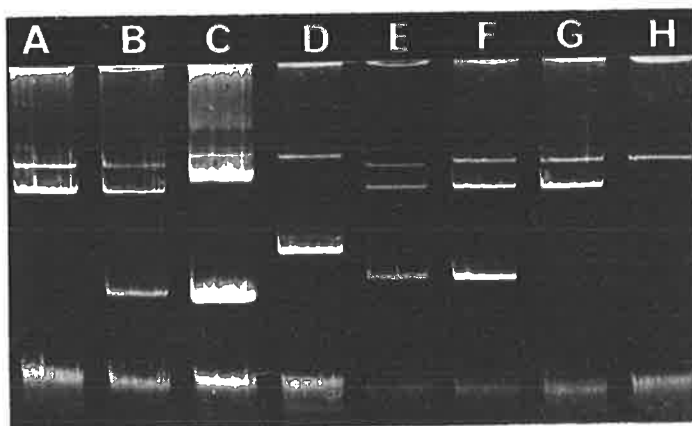

PLATE 1. Agarose gel electrophoresis of plasmid DNA.

A, (590Noc^{c} × 398RifStr) × C58C1Ch1; avirulent, non-bacteriocinogenic.
B, (590Noc^{c} × 398RifStr) × C58C1Ch1; avirulent, bacteriocinogenic.
C, 398RifStr; bacteriocinogenic.
D, C58C1 RP4.
E, K84Noc^{c}; bacteriocinogenic.
F, K84Noc^{c} × C58C1RifStr; bacteriocinogenic.
G, K84Noc^{c} × C58C1RifStr; non-bacteriocinogenic.
H, C58C1; non-bacteriocinogenic.

Migration is from top to bottom. The diffuse band of highest mobility in each track is chromosomal fragments. The agrocin plasmids are the plasmid bands of highest mobility in tracks B, C, E and F. Their mobility can be compared with that of RP4 (mol. wt34 × 10^{6} daltons [*17*]), the band of highest mobility in track D.

PLATE 2. Biological control of pathogenic strain 57 (pTi27) by bacteriocinogenic transconjugants, tested on tomato stems at a ratio of 1 : 1.

A, 57 (pTi27) + sterile water.
B, 57 (pTi27) + (84Noc^{c} × C58-C1RifStr # 1).
C, 57 (pTi27) + (84Noc^{c} × C58-C1RifStr # 2).
D, 57 (pTi27) + sterile water.
E, 57 (pTi27) + (84Noc^{c} × θ5RifStr # 1).
F, 57 (pTi27) + (84Noc^{c} × θ5RifStr # 2).

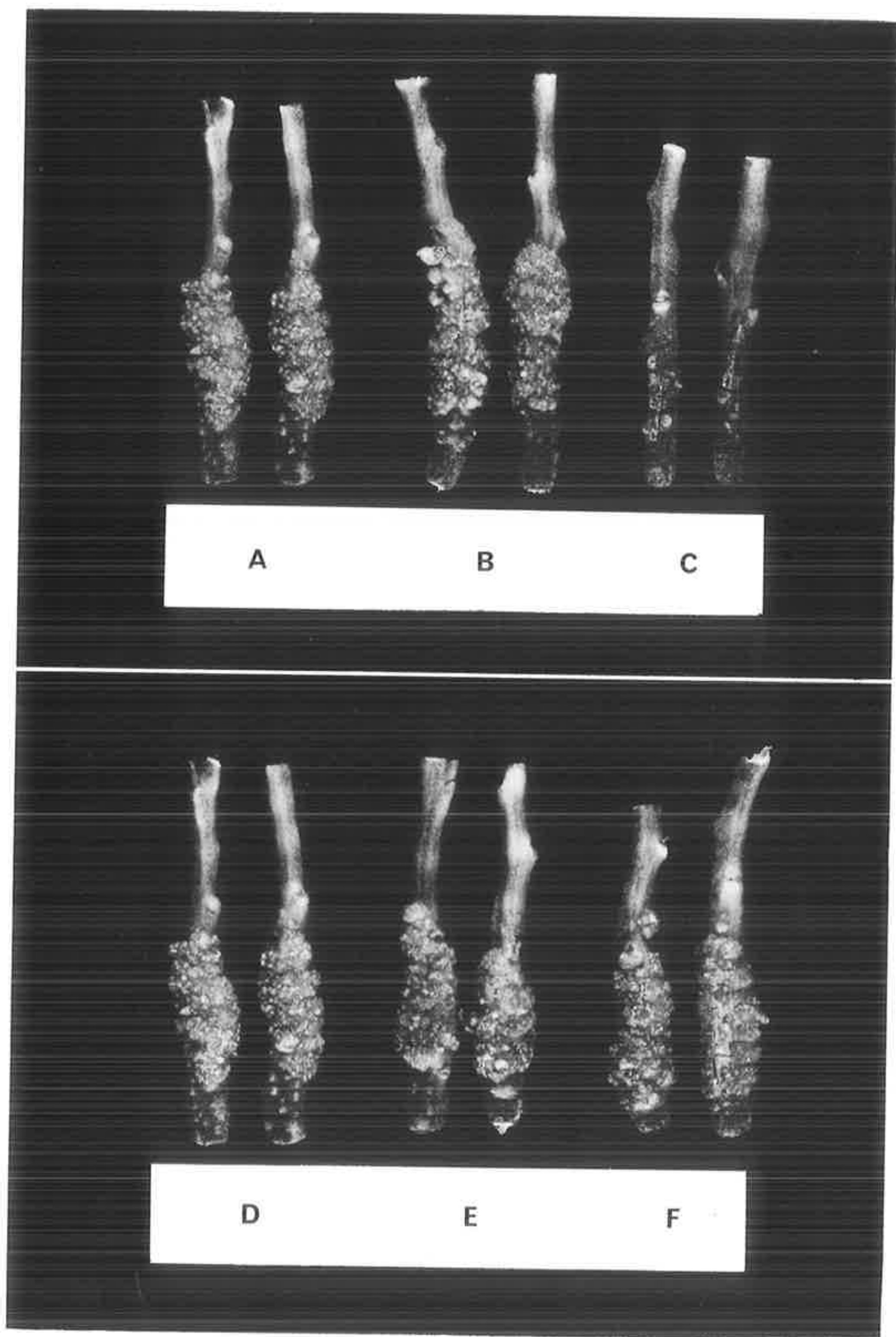

PLATE 2

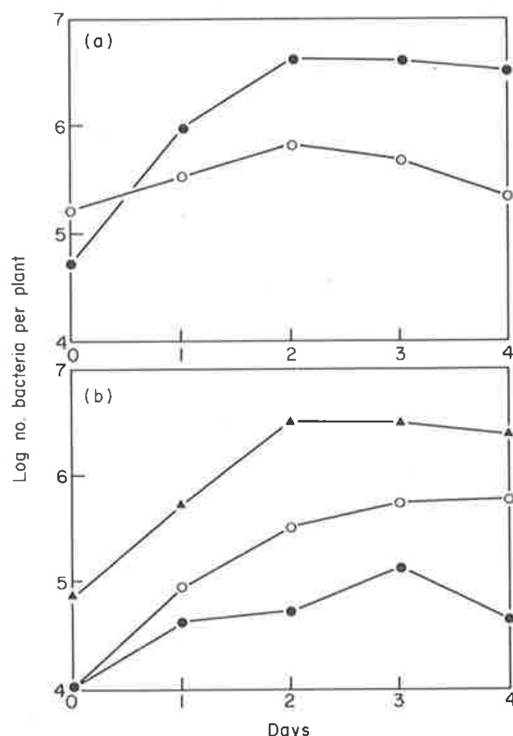

FIG. 1. Number of cells of pathogenic strain 27 and of bacteriocinogenic transconjugants following inoculation into tomato stems. (a) Bacteriocinogenic transconjugants 84Noc^c × C58-C1RifStr#2 (●) and 84Noc^c × θ5RifStr#2 (○) inoculated separately. (b) Strain 27 inoculated alone (▲) and with bacteriocinogenic transconjugants 84Noc^c × C58-C1RifStr#2 (●) and 84Noc^c × θ5RifStr#2 (○).

occurred when the two strains were mixed before inoculation. Poor growth of the ineffective strain in the inoculation sites seems the most likely explanation for its failure in biological control.

When the avirulent transconjugant no. 1 from the cross 590Noc^c × 398RifStr was tested as a biological control agent by the same method, it did not prevent crown gall induction. However, when it and the other ineffective transconjugant were tested against the pathogenic strain 198 at a ratio of 10 : 1 (producer to pathogen), crown gall induction was prevented. It would appear that lack of vigour can be compensated for by increasing the initial numbers of the agrocin producers.

One of the few Australian pathogenic field isolates that is not subject to biological control by strain 84 is strain 108 [*13*]. Although sensitive to agrocin 84, this strain produces agrocin 108 which inhibits strain 84 and many other biotype 2 strains. However, agrocin 108 does not inhibit any biotype 1 strain. So the bacteriocinogenic transconjugants (which are biotype 1) are resistant and could perhaps be used to control strain 108. Transconjugant no. 2 from the cross 84Noc^c × C58-C1RifStr was tested but proved quite ineffective at a 1 : 1 or a 10 : 1 ratio of producer to pathogen. There is no obvious explanation for this failure.

DISCUSSION

Our results support the proposal that biological control of crown gall is due to the production of agrocin 84 by strain 84 [*13*]. We have shown that the ability to produce agrocin 84 is coded for by a small plasmid. When this plasmid was transferred to suitable recipients, the transconjugants produced agrocin 84 and were able to act as biological control agents in glasshouse tests. This result is confirmed by the work of Panagopoulos *et al.* [*20*] who have isolated avirulent agrocin 84 producing strains that have presumably arisen after *in planta* conjugation between strain 84 and various recipient strains. These strains too were effective biological control agents.

Our results show that agrocin 84 production is not the only requirement for biological control. Two strains that produce agrocin 84, yet were not very effective control agents, have been described. Poor growth in wounded tomato stems is suggested as the reason for failure of these strains to control crown gall induction. A biological control agent must therefore have two properties. It must be able to produce an effective antibiotic substance and to grow and produce enough of the antibiotic to inhibit the pathogen at the surface of the potential host. For a particular situation it might be difficult to find new potential biological control agents that combine both these requirements.

The chances of success must be greatly increased if the ability to produce the effective antibiotic can be transferred by genetic manipulation to bacteria well adapted for growth at the root surface of the host plant. Various promiscuous plasmids with wide host range are available which could be used to mobilize antibiotic production. For example, the R factors such as R68.45 with its high potential for mobilization of chromosomal genes [*9*] and RP4 which can mobilize other plasmids [*3, 28*], 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.

We wish to thank Mr D. Cartwright for excellent technical assistance and Mr M. de Block for his part in developing the plasmid electrophoretic method.

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