MOLECULAR BIOLOGY AND BIOCHEMISTY OF A NOVEL CONJUGATION FACTOR IN *AGROBACTERIUM*

BY

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TABLE OF CONTENTS

SUMMARY

A previously unknown factor (Conjugation Factor or CF) responsible for controlling conjugal transfer of Ti plasmids in *Agrobacterium* has been discovered. It is a small, diffusible, hydrophobic and highly active molecule. CF is induced by opines and acts as a second messenger to induce the Ti plasmid transfer (*tra*) genes.

Wild type octopine strains cannot produce CF in shaking liquid medium but do so on solid agar plates. It was found that CF production was inhibited by excessive oxygen, forming oxygen radicals in the presence of iron.

CF was purified and identified as *N*–β-oxo-octanoyl-L-homoserine lactone. To verify this structure CF was chemically synthesised. The synthetic conjugation factor gave the characteristic IR, NMR, mass spectra and specific biological activity of the natural compound.

A series of CF derivatives were synthesised and tested for their biological activities. The results indicate that both the length and the nature (saturated or unsaturated) of the acyl chain are very important to specify biological activity.

The conjugation factor is structurally very similar to the autoinducer of bioluminescence in a very dissimilar bacterium, *Vibrio fischeri*. This striking finding suggests that there is a conserved group of N-acyl-homoserine lactone signal molecules in the microbial kingdom, wherein the length and the nature of the lipophilic acyl chain determine the biological function that is regulated.

The genes involved in the regulation and biosynthesis of the conjugation factor have been investigated. As part of this study a rapid procedure for purification of Ti plasmid DNA from *Agrobacterium* has been developed. This procedure is simple, fast and provides 8 times better yield over the traditional CsCl-EtBr density gradient ultra centrifugation method.

A total of 12 Tra$^-$ (Ti plasmid conjugal transfer deficient) and 4 Occ$^-$ (octopine catabolism deficient) mutants of *A. tumefaciens* octopine strain A6 were

isolated following Tn*5* random mutagenesis. Both of these two types of mutant can be divided into 2 groups based on whether their Tra$^-$ phenotype can or cannot be rescued by external CF.

By using the fragments adjacent to the Tn*5* insertions of relevant Cfs$^-$ mutants as hybridisation probes, three CF biosynthesis genetic regulatory loci have been identified and subcloned using deletion and complementation analyses; two are located in the Occ/Tra region of pTiA6 and one in chromosomal DNA of strain A6.

It is likely that the Occ regulatory gene, occR (Habeeb et al., 1991; Von Lintig et al., 1991), is a common regulatory gene, positively controlling Occ, Tra and Cfs functions. Tn*5* insertional mutagenesis in the Occ region of pTi A6 produces an Occ$^-$Tra$^-$Cfs$^-$ phenotype; when the Occ region was deleted from Cfs clone pA6T140, it lost its ability to produce CF in the background of strain LBA4404 (pAL4404).

CF biosynthesis is negatively regulated by a repressor encoded by cosmid clone pA6T140. The repressor region was located within a 10 kb *Hin*dIII fragment adjacent to Occ genes. It represses CF production in strain K588 (pTiB6Tra^c), a CF biosynthesis constitutive mutant, when mated into this strain. This repression is abolished in the presence of octopine.

CF biosynthesis is also regulated by a gene, named *cfr*, contained in the chromosomal DNA of strain A6. Tn*5* insertion in this region causes a Cfs$^-$Tra$^-$Occ$^+$ (normal octopine catabolism) phenotype, but the Tra$^-$ phenotype can be rescued by external CF. Therefore, *cfr* is only acts on CF biosynthesis. This gene encodes a regulatory function, because some constitutive mutants that do not have this gene can produce CF.

Conjugation factor biosynthesis function is encoded by two (or more) different *cfs* loci on pTiB6Tra^c. The *cfsA* gene was subcloned and mapped in the Occ/Tra region of the octopine-type Ti plasmid by deletion analysis. The *cfsB* locus is likely contained within or near the Vir region of the Ti plasmid.

Conjugation factor production by strain K588 (pTiB6Tra^{C}) is increased by about 4 to 5 times when about 5 copies of *cfsA* gene were introduced to that strain by mating. It appears likely that *cfsA* is a structural gene of the conjugation factor.

Ti plasmid conjugal transfer between bacteria and T-DNA transfer from bacterium to plant are very likely to have totally different mechanisms. Efficient Ti plasmid transfer occurs at 34° C and over a range of pH from pH5.6 to pH7.7, with an optimum at pH7.0. In contrast, *vir* gene induction which is a prerequisite for T-DNA transfer, is sensitive to temperatures above 30° C (Alt-Mörbe et al., 1988; Alt-Mörbe et al., 1989) and has a much more restricted pH range, with an optimum at pH5.7 (Rogowsky et al., 1987). Some chemicals such as acetosyringone and phosphate that can significantly influence *vir* gene induction (Stachel et al., 1985; Winans et al., 1988; Winans, 1990) have no detectable effect on Ti plasmid conjugal transfer; and *vice versa*, Tra$^-$, Cfs$^-$ and Occ$^-$ mutants show no observable difference in tumorigenesis on tomato plants when compared with their parent strain A6.

DECLARATION

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

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Lianhui Zhang

16/3/1983

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CHAPTER 1. GENERAL INTRODUCTION

The following review covers literature up to 1990 when the research project described in this thesis commenced.

1.1 The Genus ***Agrobacterium***

The genus *Agrobacterium* is a member of the Rhizobiaceae. It shares this position with *Rhizobium*, *Bradyrhizobium*, and *Phyllobacterium* (Jordan, 1984). *Agrobacterium* is also thought to be related to Rickettsiae (Weisburg et al., 1985) and to plant mitochondria (Yang et al., 1985) based on 16S RNA homology. The characteristics of the genus *Agrobacterium* are: single-celled, non-sporing rods; motile by means of reduced peritrichous flagella; gram negative; oxidase positive; and oxidative (Kerr and Brisbane, 1983).

Based on its phytopathological characteristics, *Agrobacterium* has been conventionally divided into five species: *A. radiobacter, A. tumefaciens, A. rhizogenes, A. rubi,* and *A. vitis*. *A. radiobacter* is nonpathogenic; *A. tumefaciens* induces crown gall on a wide range of host plants; *A. rhizogenes* induces hairyroot; *A. rubi* and *A. vitis* induce cane gall on Rubus spp. and grapevine respectively (Starr, 1946; Kersters and De Ley, 1984; Ophel and Kerr, 1990).

How this organism causes disease has been studied in depth over the past several decades.

1.2 Crown Gall Disease

Crown gall is a neoplastic disease of dicotyledonous plants caused by *Agrobacterium tumefaciens*. The large tumorous galls that result from autonomous cell proliferation are associated with the stable covalent integration of part of a large plasmid (the tumour-inducing or Ti plasmid) of the inducing bacterium into plant nuclear DNA.

Crown gall is responsible for considerable crop losses worldwide (Panagopoulos, et al., 1979; Alconero, 1980; Kerr, 1980). In Australia alone, crown gall was estimated to cause an annual loss of about A$ 1.5 million (Kerr and Brisbane. 1983). More than six hundred plant species of dicotyledons and gymnosperms are susceptible to crown gall (De Cleene and De Ley, 1976). *A. tumefaciens* can also infect a few monocotyledonous plants (Hernalsteens et al., 1984; Hooykaas-van Slogteren et al., 1984; De Cleene, 1985; Bytebier et al., 1987; Graves and Goldman, 1987), but not the economically important cereals.

Wounding of plant tissue and a suitable environmental temperature are essential for crown gall induction. It was shown that tumours only developed if tissues were inoculated with agrobacteria within a few days of wounding (Riker et al., 1946; Braun, 1952). In addition, a temperature below 30° C is essential for crown gall induction (Riker, 1926). But if the transformation process is allowed to go to completion during a 4- to 5-day period below 28° C, the transformed plant cells can develop tumours at temperatures above 30° C (Braun, 1947; Braun and Mandle, 1948).

Agrobacterium carries three genetic components that are required for inducing crown gall. T-DNA is the mobile DNA element which is transferred from the *Agrobacterium* cell to the host plant cell and integrated into the plant genome. The virulence (Vir) region is activated by plant wound factors (Stachel et al., 1985) and provides most of the trans-acting products for T-DNA transfer. Both T-DNA and Vir regions are located on the Ti plasmid. The third genetic component of the T-DNA transfer process resides in the *Agrobacterium* chromosome. Several chromosomal loci have been shown to be directly involved in attachment of *Agrobacterium* to plant cells (Douglas et al., 1985; Cangelosi et al., 1987; Matthysse, 1987; Zorreguita et al., 1988).

1.3 Ti Plasmid And Its Function

Several decades were spent investigating crown gall induction before the ultimate discovery of the Ti (tumour inducing) plasmid. Since 1927, almost every conceivable component of the bacterial cell, including the bacteria themselves, the endoantigenic fraction of the bacteria, bacterial DNA, phages carried by the bacteria and a small RNA bacterial component, had been implicated as the tumour-inducing principle, (reviewed by Braun, 1982). In the late 1960s and early 1970s significant progress was made. Two key observations were important in leading to the final identification of the tumour-inducing principle. Kerr (1969) reported that ability to cause disease could be transferred to nonpathogenic isolates of *Agrobacterium* in a developing crown gall tumour. The other important observation was reported by Hamilton and Fall (1971). They found that *Agrobacterium* strain C58 lost its tumour initiating ability when grown at a temperature of 36° C. Three years later, it was shown by Zaenen et al. (1974) that one or more large plasmids were present in a number of crown gall-inducing strains but not in eight non-pathogenic strains of *Agrobacterium* , and they suggested that the genetic information for the tumour-inducing principle in pathogenic *Agrobacterium* strains is carried by one or several large plasmids. Soon it was shown that the loss of tumour initiating ability by strain C58 grown at 37° C was due to the loss of a large plasmid which was named the tumour-inducing (Ti) plasmid (Van Larebeke et al., 1974). By using the *in vivo (in planta)* procedures described by Kerr (1969), it was demonstrated that the acquisition of tumour-inducing ability by non-pathogenic agrobacteria was the result of Ti plasmid transfer (Van Larebeke et al., 1975; Watson et al., 1975). Thus, the relationship between tumour inducing capacity of *A. tumefaciens* and the presence of the Ti plasmid was firmly established.

Braun and Mandle (1948) were the first to suggest that a tumour-inducing principle is transmitted from the inciting bacteria to the plant host cells that acquire, in a relatively short time (about 36 h), a capacity for autonomous growth and develop into crown gall tumours.

In 1960s and early 1970s, some important works on a group of unusual compounds called "opines" provided more fundamental data to indicate that there was almost certainly transfer of genetic information from *Agrobacterium* to plant. Two arginine derivatives, octopine and nopaline were discovered in crown gall tissue (Ménagé and Morel, 1964; Goldman et al., 1969). 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 *Agrobacterium* and demonstrated that those strains that induced tumours containing nopaline could catabolise nopaline and those that induced octopine galls could catabolise octopine. So they proposed that the tumorous transformation of plant cells may result from the transfer of genetic information from the bacterium to the plant.

Several years after discovery of the Ti plasmid and its relationship to pathogenecity, it was demonstrated that part of the Ti plasmid is transferred from *A. tumefaciens* to the host plant (Chilton et al., 1977) and integrated into the nucleus (Yadav et al., 1980; Zambryski et al., 1980; and Thomashow et al., 1980). The transferred DNA (T-DNA) which is flanked by imperfect direct repeats (25 bp in length) (Simpson et al., 1982; Yadav et al., 1982; Zambryski et al., 1982) varies from 13 to 23 kb, depending on the strain of bacterium. A Ti plasmid carrying a deletion of the right border is virtually avirulent on most plant species (Holsters et al., 1980; Joos et al., 1983), whereas deletions of the left border region do not affect the tumour-forming ability of the mutated plasmid (Joos et al., 1983). Later, it was demonstrated using mutated Ti plasmids that only the 25 bp right border sequences of the T-DNA is essential for T-DNA transfer and integration (Wang et al., 1984). In a mini-Ti plasmid, the left border sequence alone was also able to promote T-DNA transfer from bacterium to plant when Vir functions were supplied (Gardner and Knauf, 1986), but the right border sequence was found to be more active than the left border sequence in promoting T-DNA transformation (Jen and Chilton, 1986).

The T-DNA region carries 7 to 13 genes (Willmitzer et al., 1982; Barker et al., 1983; Joos et al., 1983; Willmitzer et al., 1983), depending on different strains. At least three of these encode the synthesis of plant hormones including an auxin and a cytokinin (Akiyoshi et al., 1984; Schröder et al., 1984; Buchmann et al., 1985). Expression of the hormone biosynthetic loci in the transformed plant cells usually causes elevated levels of auxin and cytokinin (Akiyoshi et al., 1983; Pengelly and Meins, 1982; Van Onckelen et al., 1984) which causes plant cells to exhibit uncontrolled proliferation and tumour formation.

T-DNA also encodes genes for the biosynthesis of opines (Schell et al., 1979; Holsters et al., 1980; De Greve et al., 1982; Depicker et al., 1982; Willmitzer et al., 1983). The opines serve two important biological functions. Not only do they provide a more or less reserved nutrient supply for the tumour-inducing *Agrobacterium* strains, but a subset of opines called conjugal opines also act as an environmental signal to trigger bacterial conjugation and Ti plasmid transfer (Petit et al., 1978; Tempé et al., 1978; Klapwijk et al., 1978). Different strains of *A. tumefaciens* usually synthesize different opines. The type of opine produced is one basis for classification of the Ti plasmids of *A. tumefaciens*; designations of octopine Ti plasmid, nopaline Ti plasmid and agropine Ti plasmid are commonly used (Table 1.1).

The second part of the Ti plasmid which is necessary for pathogenicity is about 40 kb and is referred to as the virulence (Vir) region (Stachel and Nester, 1986). This region is not transferred to plant cells but is involved in the environmental sensing and processing of the T-DNA. The Vir region of nopaline strains encodes six genes: *virA*, *virB*, *virC*, *virD*, *virE*, and *virG*, but octopine strains have one more plant-inducible locus, viz., *pinF* (Stachel and Nester, 1986; Rogowsky et al., 1987; Melchers et al., 1990).

Ti plasmids also encode genes for opine catabolism (e. g. *occ*, *noc*, *agc*, *arc*, *orc*) (Firmin and Fenwick, 1978; Klapwijk et al., 1978; Petit and Tempé, 1978; Ellis et al., 1979; Sans et al., 1987) and *tra* genes for Ti plasmid conjugal transfer (Klapwijk et al., 1978; Petit and Tempé, 1978). The genes responsible for opine catabolism are located on the non-transferred portion of the Ti plasmid (Petit and Tempé, 1978; Greve et al 1981; Holsters et al., 1980).

Table 1.1 Some Ti plasmids and corresponding opines*

Plasmids (example strains)	Opines produced and catabolized	Conjugal opines
Octopine (A6, B6, Ach5, NCPPB1001, 15955)	Octopine, octopinic acid, lysopine, histopine, agropine, agropinic acid, mannopine, mannopinic acid	Octopine
Nopaline (C58, T37, H100)	Nopaline, nopalinic acid, agrocinopine A	Agrocinopine A
Agropine (Bo542, AT1)	Agropine, agropinic acid, mannopine, mannopinic acid, leucinopine, agrocinopine C	Agrocinopine C

* Source: Petit et al. (1978), Tempé et al. (1978), Ellis et al. (1982), Chang et al. (1983), Tempé and Petit (1983).

1.4 Ti Plasmid Conjugal Transfer And Its Regulation

Conjugal transfer of the Ti plasmid of *A. tumefaciens* is probably important in crown gall disease epidemics because high frequency conjugal transfer of Ti plasmid between *Agrobacterium* strains has been observed, converting non-pathogens to pathogens. About 75% of non-pathogenic strains of agrobacteria become pathogenic following superinfection of a developing tumour incited by, and containing pathogenic agrobacteria (Kerr, 1971).

Conjugal transfer of a Ti plasmid is initiated when *Agrobacterium* senses the signal molecule, conjugal opine, produced by transformed plant cells (Klapwijk et al., 1978; Petit et al., 1978; Tempé et al., 1978). This is the first example of an environmental signal induced plasmid conjugation system.

Although opine induction of conjugal transfer in *A. tumefaciens* has been known for many years, we still know very little about the process. Early work was mainly on substrate induction of Ti plasmid conjugal transfer. It was found that constitutive opine catabolic (Occ^{C}) mutants are also transfer constitutive (Tra^{C}) (Tempé et al., 1978; Klapwijk and Schilperoort, 1979; Ellis et al., 1982). This suggests a model where a repressor blocks common operator sites in the promoter region of the two loci and where the opine derepresses the operon by binding to the repressor (Petit et al., 1978). However, Klapwijk (Klapwijk et al., 1978; Klapwijk and Schilperoort, 1979) presented evidence that a single insertion mutant could produce the phenotype Occ^{-}Tra. Therefore a positive control gene might also be involved in the regulation of Ti plasmid conjugal transfer.

No critical experiments on the concentration of opine inducer has been reported. Both 0.4 mM (Hooykaas et al., 1979) and 10mM of octopine (Petit et al., 1978) were used to induce Ti plasmid conjugal transfer.

Besides conjugal opines, other factors are also involved in the regulation of Ti plasmid conjugal transfer, by unknown mechanisms. A temperature greater than 30° C has been reported to inhibit conjugal transfer of pTiB6S3 (Tempé et al., 1977). Thioamino acids methionine, cysteine and cystine were found to inhibit Ti plasmid conjugal transfer when present in the conjugal induction medium at a concentration of 50 mg/liter (Hooykaas et al., 1979). The presence of 1 mg/litre MgSO4 was reported to be essential for conjugation (Klapwijk et al., 1978).

Ti plasmid conjugal transfer is mediated by *tra* genes located on the Ti plasmid itself and possibly on chromosomal DNA as well. Recent work with a nopaline strain, identified *tra* genes in three separate loci on the Ti plasmid (Beck von Bodman et al., 1989). The combined size of the three loci was only about 9 kb which seems rather small when compared with about 40 kb in the Vir region required for T-DNA transfer (Stachel and Nester, 1986) and with the transfer region of *E. coli* F plasmid which is more than 33 kb (Willetts and Wilkins, 1984). Clearly, more work is needed to reveal the mechanism of Ti plasmid conjugal transfer in *Agrobacterium*.

1.5 General Mechanism Of Plasmid Conjugal Transfer In Other Gram-Negative Bacteria

The best studied conjugative plasmids are those classified into different incompatibility (Inc) groups in *E. coli* and *Pseudomonas* strains. All of these plasmids encode conjugative pili, and their molecular mechanisms are thought to be similar (reviewed by Willetts and Wilkins, 1984; and by Ippen-Ihler, 1989). Typically, there are about 25 or more genes that occupy about 33 kb DNA and are involved in the conjugal transfer of the F plasmid. Among them, 13 genes encode for synthesis and assembling of the conjugative pilus that is essential for recognition of recipient cells and formation of mating pairs (reviewed by Willetts and Wilkins, 1984; and by Ippen-Ihler, 1989).

Contact between donor cell and recipient cell seems to act as a "signal" to initiate conjugal transfer of F-plasmid. In the absence of this signal, conjugative DNA metabolism is not triggered in the donor cell (Kingsman and Willetts, 1978). Following the effective contact of the conjugative pili of donor cells with recipient cells, cell aggregates are formed and stabilised. F plasmid DNA is nicked at oriT, the origin of transfer, by a series of reactions dependent upon the products of F genes *traM, traY, traD,* and *traI* (Traxler and Minkley, 1987; reviewed by Willetts and Wilkins, 1984). Single stranded DNA is then processively displaced in the 5'--->3' direction and transported into the recipient. DNA synthesis is started to produce a replacement strand in the donor and a complementary strand in the recipient. When conjugal transfer is complete, the F plasmid is recircularized in the recipient cell (reviewed by Willetts and Wilkins, 1984).

It is very difficult at this stage to make a comparison of the plasmid conjugal transfer processes in *A. tumefaciens*, *E. coli* and *Pseudomonas*. The molecular mechanism of Ti plasmid conjugal transfer in *A. tumefaciens* is still unknown. But at least some differences have been established. Ti plasmid conjugal transfer is induced by environmental signals in *A. tumefaciens* (Petit et al, 1978; Klapwijk et al., 1978) while in other gram-negative bacteria, conjugation function is constitutively expressed (reviewed by Willetts and Wilkins, 1984). Besides, conjugal pili which are essential for plasmid conjugal transfer in *E. coli* and *Pseudomonas* have not been observed in *A. tumefaciens* (Kerr and Ellis, 1982).

1.6 T-DNA Transfer And Its Regulation

The mechanism of T-DNA transfer from *Agrobacterium* cell to plant cell and its regulation have been the centre of attention for more than one decade. Intensive studies during this period have not only revealed the molecular mechanism of crown gall disease, but also provided an extraordinarily useful tool for the genetic engineering of plants and for the study of plant biological functions at the molecular level (reviewed by Schell, 1978; by Zambryski et al., 1983; by Kerr 1987; by Klee et al., 1987; by Binns 1988; by Daniels et al., 1988; by Zambryski, 1988; by Zambryski et al., 1989).

For successful T-DNA transfer, *A. tumefaciens* has to become attached to wounded plant cells. This process is facilitated by the chemotactic response of *A.*

tumefaciens to the phenolic metabolites of wounded plant tissue (Parke et al., 1987; Ashby et al., 1988). Attachment is under the control of five chromosomal genes; *chvA* and *chvB* encode export and synthesis respectively of a ß-1,2-glucan which is required for attachment (Puvanisarajah et al., 1985; Zorreguieta et al., 1988; Inon and Ugalde, 1989; O'Connell and Handelsman, 1989; Cangelosi et al., 1989). Immediately after attachment, bacterial cells can be removed by washing, but soon are anchored by ß-1, 4-glucan (Matthysse, 1983), a bacterial cellulose encoded by *pscA* gene and Cel region (Thomashow et al., 1987; Robertson et al., 1988). Mutation in *att* genes also results in non-attachment (Matthysse, 1987)

Processing and transfer of T-DNA is mainly mediated by *vir* genes. The expression of *vir* genes is induced by plant phenolic exudates from wounded cells. The regulatory mechanism that controls *vir* gene expression has been elucidated by a series of elegant experiments by Stachel and his colleagues (Stachel et al., 1985; Stachel and Nester, 1986; Stachel and Zambryski. 1986a). They found *vir* expression was induced by plant signal molecules, acetosyringone and hydroxyacetosyringone that are synthesized in wounded plant tissues. The induction is mediated by *virA*/*virG*, a positive regulatory system. In this system, *virA* functions as a chemoreceptor to sense the presence of plant wound factor and transmits this environmental signal to *virG* (Leroux et al., 1987). But how *virG* transcriptionally activates the other *vir* genes is still unknown. Except for *virA*, which is constitutively expressed, the other *vir* genes are normally expressed at intermediate (*virG*) or very low (*virB, virC, virD, and virE*) levels and show a 13- to 120- fold increase in gene expression following induction by plant wounding metabolites (Stachel and Nester, 1986; Stachel et al., 1986). The products of *virD* act as the T-DNA border endonuclease and are required for the production of border nicks and T-strands (Alt-Moerbe et al., 1986; Stachel et al., 1987). The level of T-DNA processing is enhanced by the product of *virC* by binding to the overdrive sequence adjacent to the right border of the T-DNA (Toro et al., 1989). In the bacterial plasmid conjugal transfer system, the transferred single strand DNA is believed to be coated with a single-strand DNA-binding protein. Similarly, it has been shown that *virE* encodes a single stranded DNA binding protein which binds to the T-strand (Christie et al., 1988; Citovsky et al., 1988). The products of *virB* are fractionated to the cell envelope, suggesting that they play a role in directing T-DNA transfer events which occur at the bacterial cell surface (Engstrom et al., 1987; Kuldau et al., 1990). In octopine strains, the plant-inducible locus *pinF* was found to encode cytochrome P-450 enzymes (Kanemoto et al., 1989), but the function of these enzymes in tumorigenesis is still unknown. The virulence of *pinF* mutants was found to be attenuated on several plants (Melchers et al., 1990; Kanemoto et al., 1989). However, in contrast to most *vir* genes, mutations in *pinF* have not been observed to completely abolish the ability of *A. tumefaciens* to form crown galls (Stachel and Nester, 1986).

The second type of *vir* gene regulation that has been reported is mediated by chromosomal DNA region. Mutations in *ros* gene lead to a 20- to 170-fold increase in the expression of *virC* and *virD* from pTi C58 or pTiA6 (Close et al., 1987). The other two chromosomal genes *chvD* and *chvE* , are also somehow involved in the regulation of *vir* gene expression. Transposon insertion in these two genes reduce *vir* gene induction significantly and strongly attenuate virulence (Winans et al., 1988; Huang et al., 1990). The molecular mechanism of this second type of regulation remains unknown.

Besides acetosyringone and its derivatives, some other environmental factors are also involved in the control of expression of *vir* genes. Acidic pH (Bolton et al., 1986; Stachel and Zambryski 1986a; Rogowsky et al. 1987), a temperature below 28° C and sucrose (or another easily metabolized sugar or glycerol) (Alt-Mörbe et al., 1988; Alt-Mörbe et al., 1989; Ankenbauer and Nester. 1990), and phosphate starvation (Winans, 1990) are required for *vir* gene induction.

A DNA conjugation model was proposed to explain the mechanism of interkingdom T-DNA transfer (Stachel and Zambryski, 1986b). Indeed, except for conjugal pili, many features of T-DNA transfer are very similar to the F plasmid conjugal transfer system in *E. coli*.. For example, contact between donor and recipient cells is essential for both processes, and border nicks of T-DNA are equivalent to nicks at the oriT of F plasmid. Furthermore, like oriT of the F plasmid, the right T-DNA border repeat is essential and functionally polar in directing DNA transfer (reviewed by Willetts and Wilkins, 1984; by Stachel and Zambryski, 1986b; and by Zambryski, 1988). One strong piece of evidence for this interkingdom T-DNA conjugal transfer model is that some derivative plasmids of pRSF1010, an *E. coli* conjugal plasmid, can be transferred from *Agrobacterium* to plants if provided with an intact Ti plasmid Vir region, an intact *oriT* and a functional Mob region of pRSF1010 (Buchanan-Wollason et al., 1987).

1.7 Possible Relationship Between T-DNA Transfer And Ti plasmid Conjugal Transfer

Both T-DNA transfer and Ti plasmid conjugal transfer processes are mediated by *Agrobacterium*. Evidence indicates that the basic processes of these two DNA transfers are similar; both involve activation of gene expression in response to external stimuli (Petit et al., 1978; Stachel et al., 1985) and, presumably, conjugal transfer of single-stranded DNA from a donor cell to a recipient cell (reviewed by Willetts and Wilkins, 1984; by Stachel and Zambryski, 1986b; by Zambryski et al., 1989). More direct evidence suggests that an evolutionary relationship may exist between T-DNA transfer and Ti plasmid conjugal transfer. A temperature greater than 30° C has been reported to inhibit bot *vir* gene induction (Alt-Mörbe et al., 1989) and Ti plasmid conjugal transfer (Tempé et al., 1977). Recently, it was reported that conjugal opines can stimulate induction of *vir* genes of the *A. tumefaciens* Ti plasmid (Veluthambi et al., 1989). Mutation of the genes *virA, virB, virC, virE,* and *virG* of the *A. tumefaciens* Ti plasmid was found to cause a 100- to 10,000-fold decrease in the frequency of conjugal transfer of Ti plasmid between *Agrobacterium* cells (Gelvin and Habeck, 1990; Steck and Kado, 1990). However, some transposon mediated Tra^-

mutants remain virulent in plants (Holsters et al., 1980; Klapwijk et al., 1978; Beck von Bodman et al., 1989; Koekeman et al., 1979).

The balance of the available evidence seems to indicate that the two DNA transfer systems are different (Beck von Bodman et al., 1989; reviewed by Kerr and Ellis, 1982), but a similar mechanism, perhaps involving the same or idiotypic proteins, may be employed both for the transfer of Ti plasmids between *Agrobacterium* strains and for the transfer of T-DNA to plant cells (Steck and Kado, 1990).

1.8 Aim And Scape Of This Study

The main aim of this study is to characterise the molecular and biochemical mechanisms of Ti plasmid conjugal transfer in *Agrobacterium tumefaciens*. The possible relationship of T-DNA transfer and Ti plasmid conjugal transfer mediated by *A. tumefaciens* is also investigated.

This thesis is divided into 9 Chapters. Chapter 1 and Chapter 9 are the introduction and general discussion respectively.

Chapter 2 deals with the investigation of the external factors that affect Ti plasmid conjugal transfer in *Agrobacterium* octopine strains with a view to providing information for selection of suitable strains for further studies, setting up a suitable conjugation bioassay and to compare the two DNA transfer processes..

Chapter 3 describes the discovery of a novel diffusible conjugation factor produced by some Tra^{e} (transfer efficient) *Agrobacterium* strains. The possibility that the conjugation factor activates or derepresses *tra* genes is discussed.

Chapter 4 focuses on the purification and characterisation of the conjugation factor. Its identity with *N*-ß-oxo-octanoyl-L-homoserine lactone is demonstrated. The possibility that conjugation factor-like molecules form a conserved group of signal molecules for gene regulation is proposed.

Chapter 5 reports that conjugation factor biosynthesis is also regulated by oxygen radicals which explains why wild type octopine strains cannot produce conjugation factor in shaking liquid cultures.

A simple and efficient method for the purification of Ti plasmids from agrobacteria is presented in Chapter 6. The method greatly facilitated the molecular biological studies described in Chapters 7 and 8.

Chapter 7 describes the molecular cloning and subcloning of a *cfs* locus which encodes part of the conjugation factor biosynthesis function. It is proposed that the Vir region of the Ti plasmid might encode a second *cfs* locus.

Chapter 8 describes the molecular mapping of *tra* (conjugal transfer) and *cfs* genes by transposon mutagenesis; three genetic regulatory loci have been identified by deletion analysis and complementation of the mutants. Conjugation factor biosynthesis is shown to be regulated by both negative and positive regulators.

CHAPTER 2. FACTORS AFFECTING TI PLASMID CONJUGAL TRANSFER

INTRODUCTION

There are two DNA transfer processes associated with the Ti plasmid of *Agrobacterium tumefaciens*; one is T-DNA transfer from *Agrobacterium* to a host plant cell (reviewed by Zambryski, 1988; Zambryski et al., 1989) and the other is Ti plasmid transfer from *Agrobacterium* donor cells to recipient cells (Petit et al., 1978; Tempé and Petit, 1978; reviewed by Kerr and Ellis, 1982). Interestingly these two DNA transfer processes are closely related by their interactions with plants; opines produced by plant cells as a result of T-DNA transfer, induce conjugal transfer of Ti plasmids between strains of *Agrobacterium* and also serve as more or less conserved food for agrobacteria carrying Ti plasmids (Klapwijk et al., 1978; Petit et al., 1978; Tempé and Petit, 1978; Ellis et al., 1982).

The genetic organisation of T-DNA transfer has been well studied (reviewed by Binns, 1988; Zambryski, 1988), but much less is known about the Ti plasmid transfer system. Characterisation of the mechanism of conjugation and Ti plasmid transfer would help to give a better understanding of the interactions between *Agrobacterium* and plants and the ecology of the Ti plasmid; it would also help to elucidate features of T-DNA transfer which was assumed to occur in a way similar to bacterial conjugation (Stachel and Zambryski, 1986; Zambryski et al., 1989).

Octopine strains were selected for this study because the conjugal inducer, octopine is commercially available. Besides, some relatively detailed physical maps of pTiAch5 (De Vos et al., 1981; Engler et al., 1981; Ooms et al., 1980), pTiA6 (Knauf and Nester, 1982) and pTiB6S3 (De Greve et al., 1981) have been published and this would facilitate genetic mapping and cloning of transfer (*tra*) genes. In this chapter, some external factors affecting Ti plasmid transfer efficiency in *A. tumefaciens* octopine strains have been investigated with a view to obtaining a better understanding of the regulation of *Agrobacterium* Ti plasmid transfer and to provide information for developing a bioassay system to screen for transfer deficient (Tra$^-$) mutants.

Two recent reports indicates that *vir* genes can influence conjugal transfer of Ti plasmid in *Agrobacterium tumefaciens* (Gelvin and Habeck, 1990; Steck and Kado, 1990). Therefore, factors that influence *vir* gene induction were also tested for their effect on Ti plasmid conjugal transfer.

MATERIALS AND METHODS

Bacterial Strains And Media

The bacterial strains used in this study are listed in Table 2.1. Strain K749, a plasmidless derivative of strain C58 was used as the recipient strain in all experiments. Strains were maintained on yeast mannitol (YM) medium (Ellis et al., 1982) and if necessary, supplemented with antibiotics.

The minimal salt medium of Petit and Tempé (1978) was the basis of induction and selective media; 200 µg/ml octopine and 1 mg/ml glucose were added except where otherwise stated. The selective medium was supplemented with 50 µg/ml rifampicin and 500 µg/ml streptomycin. The induction and selective media were at pH 7.0, except when the effect of pH on conjugation was investigated; in that experiment, the pH was varied by changing the proportion of potassium dihydrogen orthophosphate to di-potassium hydrogen orthophosphate.

TABLE 2.1 Bacterial strains and plasmids used in experiments described in this chapter

Strain	Plasmid	Description*
A6	pTiA6	Octopine catabolic, supplied by G. Morel.
NCPPB1001	pTiNCPPB1001	Octopine catabolic, supplied by NCPPB.
K608	pTiB6::Tn*5*	Octopine catabolic, T-DNA::Tn*5*, Tra^{ci} Chl^{r}, C58 pTi^{-} background, supplied by J. G. Ellis.
B6	pTiB6	Octopine catabolic, supplied by G. Morel.
Ach5	pTiAch5	Octopine catabolic, supplied by J. Schell.
B6S3	pTiB6	Octopine catabolic, a phage-sensitive isolate from U.V. irradiated and mitomycin C treated strain B6 (Vervliet et al., 1975), supplied by J. Tempé.
K749	–	C58 pTi^{-} pAt^{-} Rif^{r} Str^{r}, supplied by A. Kondorosi.

* Abbreviations used: Tra^{c}, conjugal transfer constitutive; Tra^{ci}, conjugal transfer partially constitutive and partially inducible; Chl^{r}, chloromycetin resistant; Rif^{r}, rifampicin resistant; Str^{r}, streptomycin resistant; NCPPB, National Collection Plant Pathogenic Bacteria, U.K.

Conjugation

(A) Drop mating: On day one, fresh cultures of donors and recipient strains grown on YM plates for 1-2 days were suspended in 5 ml sterile distilled water, adjusted to OD$_{600}$ of 0.4, and 200 µl of recipient suspension spread on to selective plates; 30 µl of each donor strain was inoculated into a culture tube which contained 1 ml liquid induction medium. Donors were incubated with shaking at 25°C and recipient plates were kept at 28°C or as otherwise stated. Induction time was 24 h except when the effect of induction time on conjugation was tested. On day two, donor strains were suspended and a 10-fold dilution series prepared; 10 µl of 10^0 to 10^{-5} dilutions were spotted in triplicate on to a lawn of the recipient growing on selective medium; 10 µl of 10^0 dilution of donor cells was spotted on selective medium as a control. To determine the number of viable donor cells, 10 µl of 10^{-5}, 10^{-6} and 10^{-7} dilutions were spotted on to YM medium plates. Plates were incubated at 28° C, then the number of donor and transconjugant was recorded after 2 and 5 days respectively. As the recipient strain cannot utilise octopine, transconjugants are easily distinguished from the sparse background growth. Transfer efficiencies of donor strains were calculated as the number of transconjugants observed per donor cell applied.

(B) Replica plating mating. On day one, a recipient strain was spreaded on selective medium as above and donors were applied as patches to solid induction medium. Both recipient and donors were incubated at 28° C for 24 h. On day two, induced donors were replica plated on to the recipient lawn. Transconjugants were observed after incubation at 28° C for 4 or 5 days.

Effect Of Phosphate On Conjugation

To determine the influence of phosphate on Ti plasmid conjugal transfer, donor bacteria were inoculated into the minimal induction medium containing different concentrations of phosphate (pH7.0), cultured for 24 h, and assayed using the drop mating procedure described above.

Octopine Utilisation

Test strains were inoculated into yeast extract broth (Van Larebeke et al., 1977) and incubated for 24h at 25° C. The bacteria were collected by centrifugation

(6000 rpm, 4° C) and resuspended in sterile water. The OD_{600} was adjusted to 0.4, and 0.5 ml of each inoculum was added to 3 ml of liquid induction medium which contained 50 μg/ml octopine. The presence of octopine in the medium was determined by the colorimetric method described by Lippincott *et al* (1973) at 1, 2, 5, 10, 15 and 24 h after inoculation.

RESULTS AND DISCUSSION

2.1 Sensitivity Of Strains To Octopine Induction

Octopine concentrations from 200 to 1400 μg/ml were tested on several strains for induction of Ti plasmid conjugal transfer. Results (Table 2.2) show that sensitivity of strains to octopine induction of conjugation was variable. Some were very sensitive; octopine at a concentration of 200 μg/per ml induced Ti plasmid transfer by strain A6 at a rate of 0.52×10^{-2} per donor cell. Other strains were much less sensitive, the transfer efficiency of strain B6S3 being only 0.39×10^{-4} at an octopine concentration of 1400 μg/per ml. Based on their sensitivity to octopine induction, these octopine strains can be divided into two groups, Tra^{e} (transfer efficient) and Tra^{ie} (transfer inefficient).

2.2 Influence Of Induction Time

The influence of time of induction by octopine on Ti plasmid conjugal transfer was pronounced. Data in Table 2.3 show that, for the Tra^{ie} strains B6, Ach5 and B6S3, induction for 24 h by 200 μg/ml octopine did not result in any Ti plasmid transfer. But when induction time increased to 72 h, transfer efficiencies increased markedly.

Tra^{e} strains are fully competent after 24 h induction by octopine. Shorter periods were tested with strain A6 and it was found that no Ti plasmid transfer occurred

TABLE 2.2 Effect of octopine concentration in induction medium on pTi transfer efficiency of octopine strains*

Strain	Concentration of octopine (µg/ml)					Classification based on sensitivity†
	0	200	400	1000	1400	
A6	<1 x 10^{-7}	0.50 x 10^{-2}	0.77 x 10^{-2}	0.83 x 10^{-2}	0.97 x 10^{-2}	Tra^{e}
NCPPB1001	<1 x 10^{-7}	NT[§]	1.14 x 10^{-2}	NT	NT	
K608	0.97 x 10^{-6}	NT	0.79 x 10^{-2}	NT	NT	
B6S3	<1 x 10^{-7}	<1 x 10^{-7}	0.33 x 10^{-6}	0.28 x 10^{-5}	0.39 x 10^{-4}	Tra^{ie}
Ach5	NT	<1 x 10^{-7}	0.98 x 10^{-5}	NT	0.27 x 10^{-3}	
B6	NT	<1 x 10^{-7}	0.57 x 10^{-5}	NT	o.14 x 10^{-3}	

* Donor strains were induced for 24 h.

† Tra^{e} = transfer efficient, Tra^{ie} = transfer inefficient.

§. NT = not tested.

after 5 h induction. However, Ti plasmid conjugal transfer was observed after 10 h induction, although transfer efficiency was very low (Table 2.3).

TABLE 2.3 Effect of time of induction by 200 µg/ml octopine on Ti plasmid conjugal transfer

Strain	Period of Induction (h)	Transfer efficiency
B6	24	$< 1 \times 10^{-7}$
	72*	0.19×10^{-3}
Ach5	24	$< 1 \times 10^{-7}$
	72*	0.35×10^{-3}
B6S3	24	$< 1 \times 10^{-7}$
	72*	0.57×10^{-5}
A6	24	0.50×10^{-2}
	10	0.16×10^{-6}
	5	$< 1 \times 10^{-7}$

*. Transfer efficiencies in control experiments without octopine were $<1 \times 10^{-7}$.

2.3 Uptake Of Octopine By Tra^{e} And Tra^{ie} Strains

The fact that Tra^{ie} strains need a higher concentration of octopine or a longer induction time than do Tra^{e} strains for efficient Ti plasmid conjugal transfer, indicates a possibility that the rate of the octopine uptake by these two types of strain might be different, and could cause the differences in Ti plasmid conjugal transfer efficiency. To test this possibility, rates of octopine utilisation by strains A6 and B6S3 were measured but no difference was detected (Fig.2.1). Clearly, there is an unknown mechanism affecting Ti plasmid conjugal transfer in these strains.

2.4 Influence Of Temperature

A temperature greater than 30°C has been reported to inhibit both *vir* gene induction (Alt-Mörbe et al., 1988; Alt-Mörbe et al., 1989;) and Ti plasmid transfer (Tempé et al., 1977). The latter was further investigated. Several strains were grown on octopine induction medium at different temperatures for 24 h before adding to a lawn of recipient cells grown for 24 h at corresponding temperatures. Results are shown in Tables 2.4 and 2.5. Temperatures from 24 - 34°C had no significant effect on conjugal transfer by strain A6. Although transfer could not be detected at 37°C, on transferring plates to 28°C, transconjugants appeared after two days (Table 2.4). Except for strain B6S3 which is temperature sensitive, temperatures higher than 30° C did not have a significant effect on Ti plasmid conjugal transfer by other octopine strains (Table 2.5).

2.5 Influence Of PH

As pH is known to have a marked influence on *vir* gene induction (Alt-Mörbe et al., 1989; Rogowsky et al., 1987; Vernade et al., 1988), the influence of pH during the induction period, on Ti plasmid transfer by strains A6 and K608 was studied. Optimum Ti plasmid transfer was at pH 7.0. However, relatively efficient transfer occurred over the range pH 5.6 to 7.7 (Table 2.6).

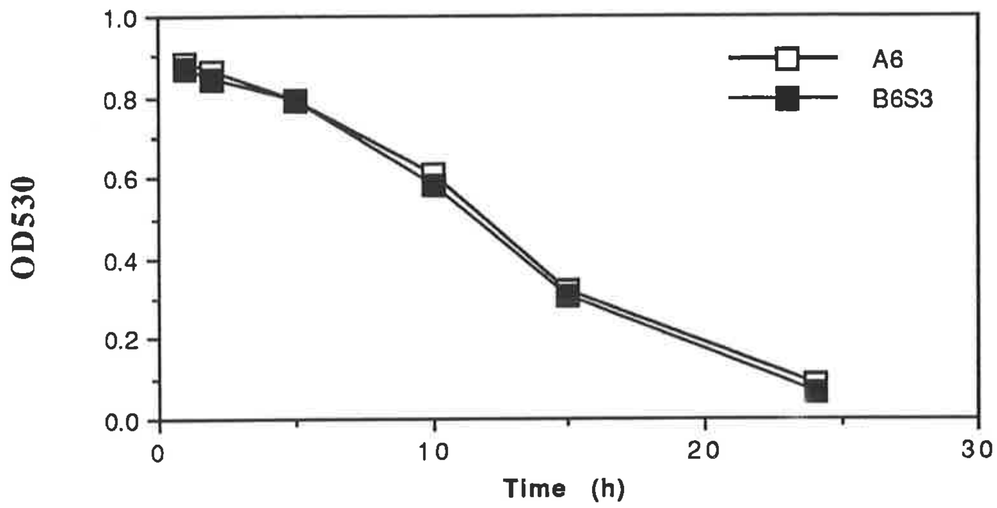


Fig.2.1 Utilisation of octopine by strains A6 and B6S3. The bacteria were grown in the octopine induction medium. The presence of octopine in the medium was determined by colorimetric method at different times after inoculation

TABLE 2.4 Effect of temperature on Ti plasmid conjugal transfer by strain A6*

Temp. °C	Transfer efficiency	
	5 days after mating	5 days after mating then 2 days at 28° C
24	0.94×10^{-2}	0.94×10^{-2}
26	0.68×10^{-2}	0.68×10^{-2}
28	0.77×10^{-2}	0.77×10^{-2}
30	1.03×10^{-2}	1.03×10^{-2}
32	0.96×10^{-2}	0.96×10^{-2}
34	0.75×10^{-2}	1.04×10^{-2}
37	$<1.00 \times 10^{-7}$	3.70×10^{-4}

* The concentration of octopine in the induction medium was 0.4 mg/ml and induction was for 24 h.

Table 2.5 Conjugal transfer efficiency of octopine strains at elevated temperatures*

Strains	Ti plasmid transfer efficiency		
	28° C	32° C	34° C
NCPPB1001	NT	0.83×10^{-2}	0.17×10^{-2}
K608	NT	0.66×10^{-2}	0.28×10^{-2}
Ach5	NT	0.14×10^{-3}	0.63×10^{-4}
B6	NT	0.24×10^{-3}	0.49×10^{-4}
B6S3	0.94×10^{-3}	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$

* The concentration of octopine in the induction medium was 2.4 mg/ml and induction was for 24 h.

TABLE 2.6 Effect of pH on Ti plasmid transfer by two octopine strains*

Strain	pH5.6	pH5.9	pH6.2	pH7.0	pH7.5	pH7.7
A6	0.39×10^{-3}	0.11×10^{-2}	0.23×10^{-2}	0.78×10^{-2}	0.29×10^{-2}	0.38×10^{-3}
K608	0.55×10^{-3}	0.15×10^{-2}	0.28×10^{-2}	0.79×10^{-2}	0.23×10^{-2}	0.37×10^{-3}

* The octopine concentration in the induction medium was 1000 µg/ml and induction was for 24 h.

2.6 Influence Of Non-Opine Chemicals

Various non-opine chemicals have been reported to influence *vir* gene induction (Stachel et al., 1985; Rogowsky et al., 1987; Winans et al ., 1988; Alt-Mörbe et al., 1989; Shimoda et al., 1990; Winans, 1990) and Ti plasmid transfer (Hooykaas et al., 1979; Klapwijk et al., 1978). Several were tested for their influence on Ti plasmid conjugal transfer by strain A6.

Results in Table 2.7 show that Ti plasmid transfer efficiency was not affected when glucose was omitted from the induction medium nor when acetosyringone was added. This is in contrast to T-DNA transfer, because expression of *vir* genes is synergisticlly controlled by acetosyringone and monosaccharides including glucose (Shimoda et al., 1990).

Transcription of the *virG* gene was reported to be partially induced by starvation for phosphate (Winans et al., 1988; Winans, 1990). But Ti plasmid conjugal transfer seems unaffected by phosphate concentrations in the induction medium ranging from 5-100 mM (Table 2.7).

The presence of 1 mg/ litre $MgSO_4$ was reported to be essential for Ti plasmid transfer of *Agrobacterium* (Klapwijk et al., 1978). Omitting $MgSO_4$ from the induction medium did not influence Ti plasmid transfer by donor strains NCPPB1001, K608, B6, B6S3 and Ach5; but no Ti plasmid transfer by strain A6 was detected in the absence of $MgSO_4$. However, strain A6 could not grow on induction medium lacking $MgSO_4$ (Table 2.7).

TABLE 2.7 The influence of various chemicals on Ti plasmid conjugal transfer by strain A6

Induction medium	Transfer efficiency
complete medium (Phosphate 100mM)	0.67×10^{-2}
phosphate at 50 mM	0.51×10^{-2}
phosphate at 5 mM	0.17×10^{-3}
minus glucose	2.99×10^{-2}
minus $MgSO_4$	$<1 \times 10^{-7}$*
minus $MgSO_4$, supplemented with 1g/l $(NH_4)_2SO_4$	$<1 \times 10^{-7}$*
plus 30mM acetosyringone	0.83×10^{-2}
plus 100mM acetosyringone	0.85×10^{-2}
plus 50 µg/ml L-methionine	0.46×10^{-6}
plus 100 µg/ml L-methionine	$<1 \times 10^{-7}$
plus 100 µg/ml bromothymolblue	0.52×10^{-3}
plus 150 µg/ml bromothymolblue	0.76×10^{-4}

* Strain A6 did not grow in induction medium.

Sulphur-containing amino acids were reported as blocking Ti plasmid transfer (Hooykaas et al., 1979). Results in Table 2.7 show that addition of 50 and 100 µg/ml L-methionine to the induction medium markedly reduced or completely prevented Ti plasmid transfer. Similar results were obtained with strain NCPPB1001 and B6S3.

Bromothymolblue at a concentration of 150 µg/ml has been used as an indicator for octopine catabolism and Ti plasmid transfer by octopine strains (Hooykaas et al., 1979). Results in Table 2.7 show that Ti plasmid transfer efficiency of strain A6 was decreased by about 100 times when 150 µg/ml bromothymolblue was added to the induction medium.

CONCLUSION

Several octopine strains of *Agrobacterium tumefaciens* were tested for Ti plasmid transfer following induction by 400 µg/ml octopine for 24 h. The strains could be divided into two groups, transfer efficient (Tra^e) and transfer inefficient (Tra^{ie}); the respective rates of transfer were 0.77 - 1.14 x 10^{-2},and 0.33 - 9.8 x 10^{-6} plasmid transconjugants per donor cell. Transfer efficiencies of Tra^{ie} strains were greatly increased when the time of induction was 72 h.

Various factors known to influence *vir* gene induction were tested for their effects on Ti plasmid transfer. A temperature of 34°C had no effect, except in one strain B6S3. Plasmid transfer occurred over a range of pH, from pH 5.6 to pH 7.7,.with an optimum of 7.0. Glucose, acetosyringone and phosphate had no significant influence on Ti plasmid transfer. It would appear that Ti plasmid transfer and T-DNA transfer following *vir* gene induction are two entirely separate processes.

CHAPTER 3. DISCOVERY OF A DIFFUSIBLE COMPOUND WHICH CAN ENHANCE TI PLASMID CONJUGAL TRANSFER

INTRODUCTION

It was shown in chapter 2 that octopine strains of *A. tumefaciens* can be placed in two distinct groups, namely Tra^{e} and Tra^{ie}, based on their conjugal transfer efficiency following induction by various concentrations of octopine. One question which arises is what causes the difference? In a preliminary experiment to investigate the effect of temperature on Ti plasmid transfer by different strains, it was noted that, under some conditions, transconjugant colonies of Tra^{ie} strains appeared only when adjacent to Tra^{e} transconjugants. This unusual phenomenon is further investigated in this chapter.

MATERIALS AND METHODS

Bacterial Strains And Media

The *Agrobacterium* strains A6, NCPPB1001, K608, B6, Ach5, B6S3 and recipient strain K749 have been described in Chapter 2. The other strains used in this study are listed in Table 3.1. The maintenance medium, induction and selective media were the same as described in Chapter 2.

Chemicals And Enzymes.

Opines used in this study were synthesized by M. E. Tate and J. Tempé or purchased from Sigma. L-proline, D-proline, L-arginine, L-ornithine, protease Type

XIV, RNase Type 1-A and DNase Type III are the products of Sigma. The enzymes were dissolved in sterile distilled water before use.

TABLE 3.1. Bacterial strains and plasmids used in the experiments described in this chapter

Strain	Plasmid	Description*
K794	pTiB6::Tn*5*	Octopine catabolic, TR-DNA::Tn*5*, Tra^c Rif^r Str^r, C58 pTi^- background, supplied by J. G. Ellis.
K804	pTiB6::Tn*5*	Octopine catabolic, TR-DNA::Tn*5*, Tra^c Rif^r Str^r, C58 pTi^- background, supplied by J. G. Ellis.
T37	pTiT37	Nopaline catabolic, Tra^{ci}, supplied by G. Morel.
K323	pTiT37	Nopaline catabolic, Tra^c colony of strain T37 isolated from octopine medium, supplied by A. Kerr.
K749(pTiK608)	pTiB6::Tn*5*	Octopine catabolic, transconjugant from K608 x K749, this study.
K608C1	–	pTiB6::Tn*5* was cured from strain K608, this study.

* Abbreviations used: Tra^c, conjugal transfer constitutive; Tra^{ci}, conjugal transfer partially constitutive and partially inducible; Rif^r, rifampicin resistant; Str^r, streptomycin resistant.

Conjugation

Drop mating and replica plate mating were carried out as described in Chapter 2.

Conjugation Factor Preparation And Activity Bioassay

Conjugation factor (CF) filtrate was prepared as follows: strain K608 was inoculated into 10 ml of liquid induction medium which contained 400 μg/ml octopine and incubated at 25°C for 24 h, with shaking. The culture was centrifuged and the supernatant collected and sterilised by membrane filtration (0.2 μm, Millipore). Presence of octopine was determined. When testing CF induction by other opines and chemicals, they also were used at 400 μg/ml, and the medium, plus additive was used as a control to make sure that enhanced Ti plasmid transfer efficiency was due to CF rather than to those opines and chemicals.

The same procedure was used to examine the CF producing ability of all Tra^{c} and Tra^{ci} (partially constitutive for transfer) strains used. For the inducible strains A6, NCPPB1001, B6, Ach5, K749, and plasmidless strain K608C1 which fail to produce CF in liquid medium, the replica plate mating method was used to determine CF production; the CF producing strain and CF activity indicator strain (B6S3) were patched side by side on the induction medium at a distance of 1.2 cm (refer to Fig.3.2).

Strain B6S3 was selected to indicate CF activity because it cannot transfer the Ti plasmid when the concentration of octopine in the induction medium is 200 μg/ml (Chapter 2). Drop mating and replica plate mating methods were used respectively in quantitative and qualitative assays of the promoting effect of CF on Ti plasmid conjugal transfer. In the qualitative assay, 400 μl of a CF preparation was added to an agar induction plate containing 200 μg/ml octopine; after drying, strain B6S3 was patched onto the plate, incubated for 24 h at 28°C and replica plated on to a lawn of recipient on selective medium. In the quantitative assay, 200 μl of CF filtrate and 30 μl of a cell suspension of strain B6S3 were added to 1 ml liquid induction medium (200μg/ml octopine), incubated for 24 h, diluted and 10 µl drops added to a lawn of recipient on selective medium. The number of viable donor cells was determined as described in Chapter 2.

Aggregation Assay.

The method of Dunny *et al* (1978) was followed to determine wether CF could induce aggregation between donor cells, recipient cells, or combined donor and recipient cells; 1 ml of CF preparation was mixed with 800 µl of fresh liquid induction medium plus 200 µl of late log phase cultures of donor cells, recipient cells, or 1:1 mixture of donor and recipient, incubated at 28°C, and monitored for aggregation under a compound microscope (8 x 8 magnification).

RESULTS AND DISCUSSION

3.1 Detection Of A Novel Diffusible Conjugation Factor (CF).

Tra^{ie} strain B6S3 cannot transfer its Ti plasmid when octopine concentration in the induction medium is 200 mg/litre (Chapter 2). But when strain B6S3 and Tra^{e} strains A6 and NCPPB1001 were patched side by side on the induction plates and cultured for 24 h before mating, some transconjugant colonies of Tra^{ie} strains appeared on the selective plates but only when adjacent to Tra^{e} transconjugants. It appeared that Tra^{e} strains were producing a diffusible conjugation factor (CF) which could stimulate Ti plasmid transfer (Fig.3.1). This was confirmed by a "ditch plate" experiment in which a Tra^{ie} strain was separated from a Tra^{e} strain on an agar induction plate by removing a thin slice of agar. Fig.3.2 shows the effect of chemical diffusion from strain K608 to strain B6S3. When the agar slice was not removed, transconjugant colonies of strain B6S3 appeared only when adjacent to strain K608. When the two strains were separated

Fig.3.1. A diffusion phenomenon observed in conjugation selective medium. The *Agrobacterium* donor strains NCPPB1001 (A), A6 (B) and B6S3 (C) were patched onto octopine induction medium (left) and incubated for 24 h and then replica-mated with recipient strain on selective medium (right). Transconjugant colonies of Tra^{e} strains NCPPB1001 and A6 are shown on the top half of the selective plates. But transconjugant colonies of Tra^{ie} strain B6S3 appeared only in the region adjacent to Tra^{e} strains.

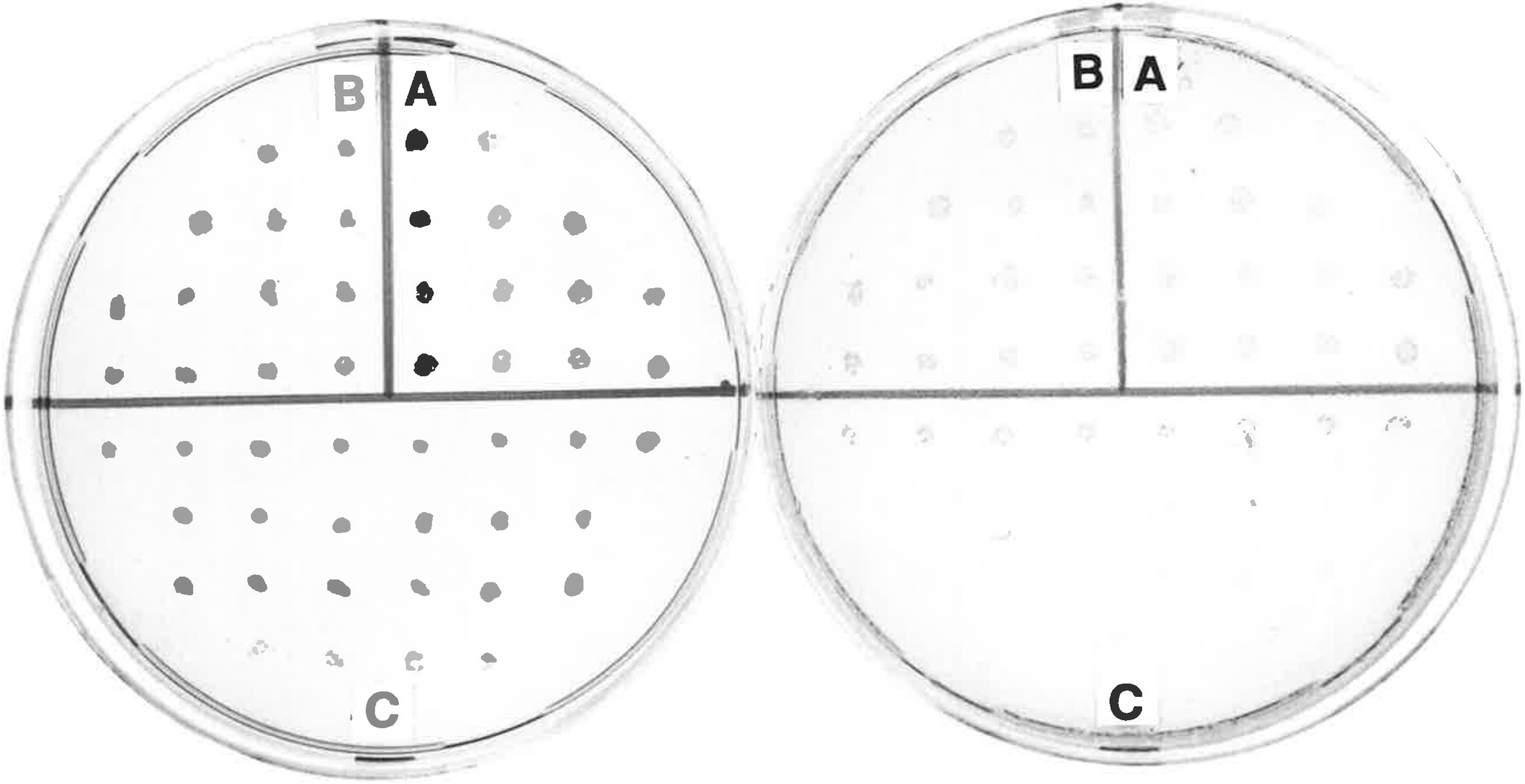
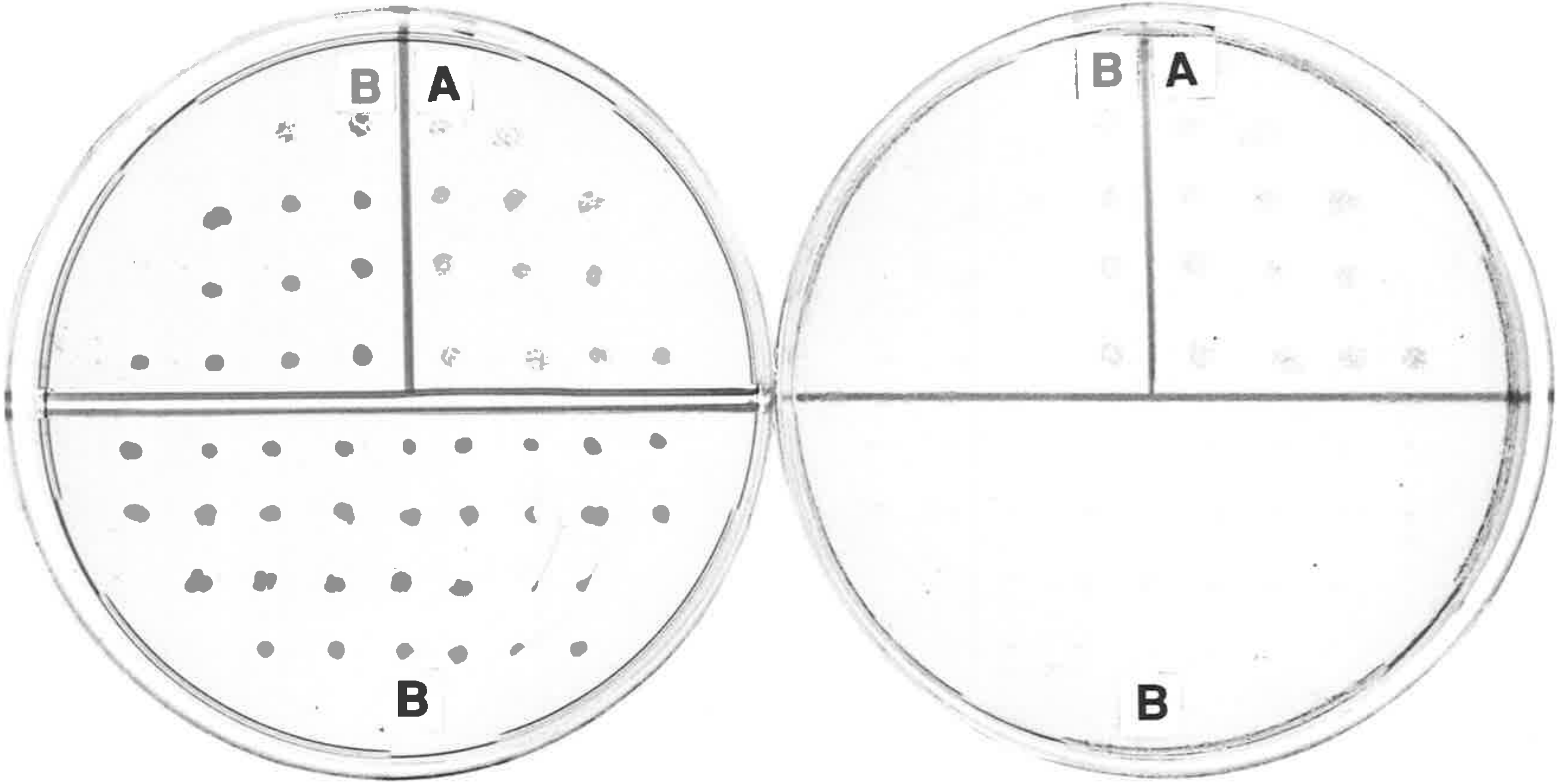

B
A
C
B
A
C

Fig.3.2 A diffusible conjugation factor produced by strain K608 (A) promotes pTi conjugal transfer of strain B6S3 (B). Left: Induction plate. Right: Selective plate. Double lines indicates the location of ditch.


B
A
B
B
A
B

by a "ditch" in the induction medium, no transconjugants of strain B6S3 could be seen on the selective medium.

The discovery of CF probably explains the difference in Ti plasmid transfer efficiency of Tra^{e} and Tra^{ie} strains.

3.2 Influence Of CF On Ti Plasmid Transfer Efficiency.

To determine the relationship between CF production and the conjugal transfer efficiency of Ti plasmids, several octopine strains were tested for synthesis of CF following induction by various chemicals for varying periods. Table 3.2 shows that there was a strong correlation between CF production and Ti plasmid conjugal transfer efficiency. Tra^{e} strains A6 and NCPPB1001 produced CF after being induced for 24 h by 200 µg/ml octopine on agar plates, but Tra^{ie} strains Ach5 and B6 could only synthesize CF after being induced for 72 h. Tra^{c} strains could produce CF constitutively but in the Tra^{ci} strain K608 (conjugal transfer partially constitutive), CF production was detected only following induction by octopine .

Conjugation factor can greatly enhance the conjugal transfer efficiency of Tra^{ie} strains. Strain B6S3 was induced by a wide range of octopine concentrations with and without the addition of CF. Fig. 3.3 shows that the presence of CF had a marked influence on conjugation over the whole range of octopine concentrations. However, when no octopine was present, CF did not induce conjugation.

3.3 *Repression of CF production by wild type* ***Agrobacterium*** *strains in shaking liquid culture*

Several strains were tested for production of CF in both liquid and solid induction media. All Tra^{c} and Tra^{ci} mutants tested (K608, K794, K804, T37 and K323) produced CF in both solid and liquid media, but with the wild type inducible strains A6, NCPPB1001, B6 and Ach5, CF production was detected only in solid medium. The difference between Tra^{c} and wild type strains is that Tra^{c} strains produce CF

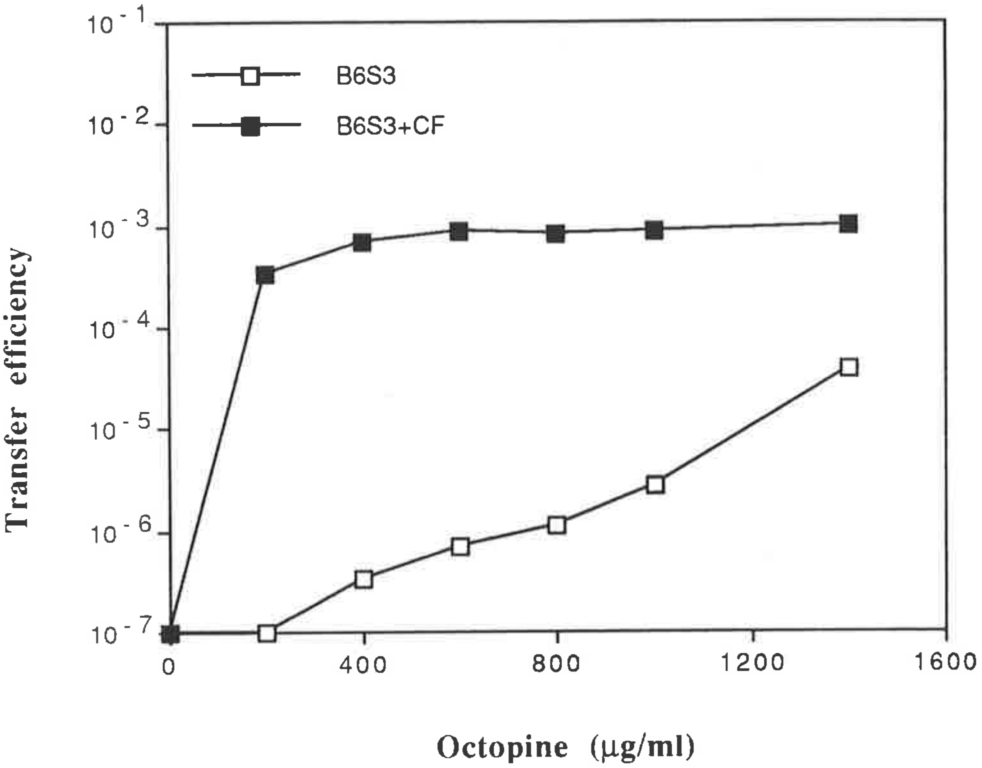


Fig.3.3 CF promoting Ti plasmid conjugal transfer by strain B6S3

constitutively, while CF production in wild type strains is inducible by octopine. Clearly, there must be an unknown factor(s) in the shaking liquid cultures that interferes with the induction of conjugation factor by octopine in wild type *Agrobacterium* strains. This phenomenon is further investigated in Chapter 5.

3.4 Relationship Of CF To Nopaline Strains.

Ti plasmids can be divided into octopine, nopaline and agropine types based upon the opines they produce and catabolize(Tempé and Petit, 1978; Kerr and Ellis, 1982). Although different opine strains need specific opines for induction of Ti plasmid conjugal transfer (Ellis et al., 1982; Kerr and Ellis, 1982; Tempé and Petit, 1983), strong homology between Tra regions of these strains has been observed by several groups (Holsters et al., 1980; Greve et al., 1981; Clare et al., 1990). This indicates that there are some *tra* genes conserved in different opine-type Ti plasmids. When the nopaline Tra^{c} and Tra^{ci} strains K323 and T37 were grown for 24 h in liquid induction medium with 1 g per litre $(NH_4)_2SO_4$, a substance was produced that promoted conjugal transfer in the octopine strain B6S3. This fact suggests that CF is probably a common factor among different opine strains.

3.5 Requirement Of Ti Plasmid For CF Production.

The Ti plasmid is required for the production of CF. When the Ti plasmid was cured from strain K608, it lost CF production ability; conversely, strain K749 gained this ability along with the Ti plasmid from strain K608, following conjugation between these two strains (Table 3.3).

TABLE 3.2 CF production by different octopine strains of *Agrobacterium* , with and without induction

Strain	Nature of transfer system*	Inducing chemical (per litre)	Induction time (h)	CF activity†
K794	$\mathrm{Tra}^{\mathrm{c}}$	1 g $(NH_4)_2SO_4$	24	+
K804	$\mathrm{Tra}^{\mathrm{c}}$	as above	24	+
K608	$\mathrm{Tra}^{\mathrm{ci}}$	as above	24	–
		200 mg octopine	24	+
A6	$\mathrm{Tra}^{\mathrm{e}}$	200 mg octopine	24	+
NCPPB1001	$\mathrm{Tra}^{\mathrm{e}}$	as above	24	+
B6	$\mathrm{Tra}^{\mathrm{ie}}$	400 mg octopine	24	–
		400 mg octopine	72	+
Ach5	$\mathrm{Tra}^{\mathrm{ie}}$	400 mg octopine	24	–
		400 mg octopine	72	+

* $\mathrm{Tra}^{\mathrm{c}}$ = constitutive for transfer; $\mathrm{Tra}^{\mathrm{ci}}$ = partially constitutive , partially inducible for transfer; $\mathrm{Tra}^{\mathrm{e}}$ = efficient transfer following induction; $\mathrm{Tra}^{\mathrm{ie}}$ = inefficient transfer following induction.

† CF production by strains K794 and K804 was determined by using a CF filtrate preparation., the other strains by using the "ditch plate" method.

3.6 Imino Acids Induce CF Production In Strain K608.

Because nopaline strains can produce a compound which promotes Ti plasmid transfer by an octopine strain, it appeared possible that CF induction also was not highly specific. A wide range of chemicals was tested using the Tra^{ci} strain K608 as indicator. Results (Table 3.4) show that all imino acids tested could induce strain K608 to produce CF although some of them could not be utilised by strain K608, or were utilised only poorly. Octopine was the most efficient inducer, followed by nopaline and noroctopinic acid. L- and D-proline which are not opines, could also efficiently induce production of CF. But L-arginine and pyruvate, the components of octopine (Ménagé and Morel, 1964) had no inducing effect.

TABLE 3.3 The influence of the Ti plasmid on CF production

Strain	Ti plasmid	Inducing chemical* (400 μg/ml)	CF production†
K749	–	octopine	-
		nopaline	-
K749(pTiK608)	+	octopine	+
		nopaline	+
K608		octopine	+
		nopaline	+
K608C1	–	octopine	-
		nopaline	-

* Induction time is 24 h.

† The bacteria were grown in liquid induction medium for 24 h. CF activity in the culture filtrates was determined using strain B6S3 as bioassay strain.

TABLE 3.4 CF production by strain K608 following induction by various imino acids

Chemical	Catabolism by strain K608	CF activity *
Octopine	+	0.83×10^{-3}
Nor~~o~~octopinic acid†	very poor	0.87×10^{-4}
Homooctopine†	–	0.61×10^{-5}
Agropine	+	0.35×10^{-4}
dMan-Glu†	poor	0.42×10^{-5}
Nopaline†	–	0.97×10^{-4}
Allo-nopaline†	–	0.36×10^{-5}
L-proline	+	0.72×10^{-4}
D-proline	+	0.54×10^{-4}
L-arginine and pyruvate	+	$< 1 \times 10^{-7}$
Control§	+	$< 1 \times 10^{-7}$

* CF activity is expressed as pTi transfer efficiency by strain B6S3.

† Where the inducing chemical could not be catabolized by strain K608, the medium was supplemented with 1 mg/ml $(NH_4)_2SO_4$ to provide a source of nitrogen; dMan-Glu is the abbreviation of N^2-(1'-Deoxy-D-mannitol-1'-yl)-L-g~~i~~utamate. /l

§ Control treatment consisted of 1mg/ml $(NH_4)_2SO_4$.

Results suggest that the imino group might be the basic structure needed for CF induction in strain K608. However at this stage, it cannot be determined whether imino acids other than octopine can also induce CF production in wild type inducible strains, because these strains do not exude CF into liquid induction medium under the culture conditions used; the other method for CF detection, viz., the diffusion agar plate method described in Fig 3.1 and Fig.3.2, is not suitable for this propose since CF needs to cooperate with octopine to initiate Ti plasmid conjugal transfer (see section 3.2).

3.7 Effect Of Temperature On CF Production By Strain B6S3.

Tempé et al (1977) found that strain B6S3 could not transfer its Ti plasmid at a temperature above 30°C. Data in Table 3.5 confirm their observation. When induction temperature was higher than 30°C, transfer efficiency of strain B6S3 was less than 1 x 10^{-7}. However, if CF was added to the induction medium, strain B6S3 could transfer its Ti plasmid at 32°C and 34°C at rates of 0.93 x 10^{-3} and 0.55 x 10^{-4} respectively.

3.8 Properties Of The Diffusible Conjugation Factor.

One ml aliquots of CF preparation were treated (a) with 2N NH_4OH at 45°C for 2 h; (b) by autoclaving at 121°C for 15 min; (c) with DNase (500 μg/ml); (d) with RNase (500 μg/ml); (e) with protease (50 μg/ml). All enzymatic treatments were at 37°C for 20 min followed by 100°C for 10 min. After treatments, 400 μl from each treatment was used to assay CF activity. Only treatments (a) and (b) removed activity. So it is clearly not a nucleic acid or protein.

CF is a small molecule; the filtrate obtained after passing CF through Centricon 10 membrane which is a microconcentrator designed to retain molecules larger than 10,000 M.W. had lost no activity. Besides, it is also dialyzable; when a CF preparation was dialyzed overnight against liquid induction medium lacking octopine, CF activity was lost from the solution within the membrane but could be detected in the external liquid following concentration.

TABLE 3.5. Ti plasmid conjugal transfer by strain B6S3 at a range of temperatures, with and without CF

Induction treatment*	Temperature °C 24	28	30	32	34	37
2.4 mg/ml octopine	1.01×10^{-3}	0.94×10^{-3}	0.37×10^{-3}	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
2.4 mg/ml octopine + CF	1.04×10^{-3}	1.12×10^{-3}	0.88×10^{-3}	0.93×10^{-3}	0.55×10^{-4}	$<1 \times 10^{-7}$

* Induction time was 24 h.

3.9 Mode Of Action.

The fact that CF can diffuse from bacterial donor cells prompted us to consider that CF might have some effect on recipient cells. Therefore, 400 µl CF preparation was added to the selective medium before adding recipient, incubated for 24 h at 28°C, then mated by drop mating with strain B6S3 which had been induced for 24 h by 200 µg/ml octopine. But the result showed no difference in transfer efficiency between that treatment and the negative control.

Dunny *et al* (1978) reported that recipient strains of *Streptococcus faecalis* produce a diffusible clumping-inducing agent which causes strains carrying certain conjugative plasmids to aggregate. But conjugation factor seems likely to be a different kind of signal molecule. It did not induce any measurable aggregation between donor cells, or recipient cells, or a mixture of donor and recipient.

Although quantitative measurement of CF concentration is impossible at this stage, there can be little doubt that CF is a highly active molecule. A 50 fold dilution of a CF preparation can still enhance Ti plasmid transfer efficiency in $\mathrm{Tra}^{\mathrm{ie}}$ strain B6S3 more than 1000 times when coinduced by 200 µg/ml octopine.

CONCLUSION

A diffusible conjugation factor (CF) was produced by *Agrobacterium tumefaciens* following induction by octopine and some other imino acids. The evidence indicates that CF is a key signal molecule affecting transfer efficiency of Ti plasmids (pTi) but is not sufficient by itself to induce transfer. $\mathrm{Tra}^{\mathrm{c}}$ mutants can produce CF constitutively and transfer efficient ($\mathrm{Tra}^{\mathrm{e}}$) strains can produce it at low octopine concentrations. Transfer efficiency of transfer inefficient ($\mathrm{Tra}^{\mathrm{ie}}$) strains was greatly increased by adding CF to the induction medium. The thermosensitive strain B6S3 which normally cannot conjugate at temperatures above 30°C, could transfer pTi efficiently at 32°C and 34°C in the presence of CF.

Production of CF is dependent on the presence of pTi but appears to be common for different opine strains; it was first detected in octopine strains but nopaline strains also produced the same or a similar compound.

CF is a small molecule, resistant to DNase, RNase, protease and 100° C for 10 min, but autoclaving (121° C for 15 min) and alkaline treatment removed all activity. CF is a highly active molecule, acting on donor cells (its producer) but not on recipient cells. It appears that CF activates or derepresses *tra* genes in *Agrobacterium.*

All Tra^c strains could produce CF in shaking liquid culture, but CF production by wild type strains was not detected in shaking liquid octopine induction medium and presumably was repressed by unknown factors.

CHAPTER 4. PURIFICATION AND CHARACTERIZATION OF CONJUGATION FACTOR

INTRODUCTION

There are two types of signal molecule mediating the conjugal transfer of Ti plasmids in *Agrobacterium tumefaciens.* A conjugal opine is an environmental signal which triggers the production of a diffusible conjugation factor (CF) in donor strains (Zhang and Kerr, 1991; Chapter 3). Conjugal transfer efficiency of the Ti plasmid in some strains of *Agrobacterium tumefaciens* is greatly enhanced by this diffusible conjugation factor produced by other strains of *A. tumefaciens* (Zhang and Kerr, 1991; Chapter 3). Transposon mediated mutants that do not produce CF, fail to conjugate unless supplied with CF in the induction medium (Chapter 8). These data indicate that CF is a key signal molecule and behaves as a secondary messenger, transmitting the environmental information to *tra* genes. It is the first example of a second messenger molecule in a bacterial conjugation system.

Clearly, characterisation of this interesting molecule is essential for a proper understanding of the regulation mechanism for Ti plasmid conjugal transfer in *Agrobacterium.* This chapter presents results of purification and characterisation of the conjugation factor.

MATERIALS AND METHODS

Bacterial Strains And Medium

CF producing strain K588, K608, K794, and K804 has been described in Chapters 2 and 3. CF bioassay strain NT1 (*traR; tra::lacZ749*) was kindly supplied by

Stephen Farrand (Piper et al., 1993). Tra^-Cfs^- strain M103 is described in Chapter 8 (Tables 8.1 and 8.2). Conjugation recipient strain K749 has been described in Chapter 2.

Solvents And Reagents

All solvents and reagents used were of analytical grade or better.

Preparation Of Concentrated Conjugation Factor Ethyl Acetate Extracts

For production of the conjugation factor, strain K794 was grown in Petit's liquid minimal medium (Petit and Tempé, 1978) for 2 days at 28° C, using proline (4 g/litre) and mannitol (10 g/litre) as the nitrogen and carbon sources. Bacterial culture filtrates (5 litre) were extracted two times by the same volume of ethyl acetate for 5 min. The organic phase was separated and concentrated at 45° C in a rotary vacuum evaporator, the residue was dissolved in 5 ml of ethyl acetate and is subsequently referred to as CF concentrate.

High Voltage Paper Electrophoresis

The method of Tate (1968) was followed for high voltage paper electrophoresis of CF. Concentrated CF extracts were loaded at the origin as thin streaks at a rate of approximately 5 µl /cm. After electrophoresis, the paper was allowed to dry, then 0.5 cm squares cut out along the run and eluted with 500 µl of 40% methanol. CF activity in the eluates was determined.

Paper Chromatography

Ascending paper chromatography was according to the method described by Block et al. (1958). Sample loading and subsequent CF activity determination were the same as described for high voltage paper electrophoresis. Solvents used for chromatography are listed in Table 4.4.

Thin Layer Chromatography

Silica plates(Kieselgel 60 F254 , 10 x 20 cm) and C18 reverse phase thin layer plates (RP-18 F254s, 5 x 10 cm) produced by MERCK Company were used in this experiment. Samples were loaded as described for high voltage paper electrophoresis. After running, the solvents were evaporated by blowing the plate with air, then 0.5 cm squares of silica or C18 gel were scraped off the plates along the run and eluted with 500 µl of methanol. CF activity in the eluates was determined. Solvents used for chromatography are listed in Table 4.5 and Table 4.6.

is it porosity?
type of membrane?

HPLC Resolution Of Conjugation Factor

HPLC was performed with a reverse phase C18 column (MERK HIBAR, Lichromosorb). The analytical column (25 cm, 4 mm i.d.) was protected by a C18 precolumn. All solvents were HPLC grade, filtered through a 0.45 mm membrane and ultrasonically degassed before use. Samples were membrane filtered (0.45 mm) before analysis. Elutions were conducted at 1 ml/min with a linear gradient of 30-45 % methanol. Peak spectra were recorded at 210-400 nm with a diode array detector and detection was at 215 nm.

0.45 µ?

Large Scale Flash Chromatographic Purification Of Conjugation Factor

Concentrated ethyl acetate CF extracts (1 ml) were applied to a 3 x 23 cm column of 60-200 mesh silica gel. The column was eluted with ethyl acetate by the method of flash chromatography (Still et al., 1978). Active fractions were identified by bioassay and pooled; they were evaporated to dryness, redissolved in 0.5 ml of 30% methanol and applied to a 1 x 15 cm C18 reverse phase column. The column was eluted with 30% v/v methanol/water by flash chromatography. The silica column and C18 reverse phase column flash chromatography were repeated five times to complete partial purification of the concentrated CF extracts from a total of 5 litres of bacterial culture filtrate. Evaporation of the combined active fractions gave a yellowish white solid (3 mg/

L culture). Further purification by C18 reverse phase HPLC gave two separate biologically active components, labelled CF1(retention time 13.38 min.) and CF2 (retention time 11.4 min).

Conjugation Factor Activity Bioassay

Initially, the CF bioassay method (conjugation method) described in Chapter 3 was used to monitor CF activity. Later, during large scale purification, CF activity was determined by induction of a *tra::lacZ749* reporter gene fusion in strain NT-1(*traR*) (Piper et al, 1993). β-Galactosidase activity was expressed as Miller units (Miller, 1972). CF solutions were added to the minimal medium (Petit and Tempé, 1978) plus $(NH_4)_2SO_4$ (0.2%,w/v) and mannitol (0.2%,w/v) and then inoculated with a 1:40 dilution of a late log phase culture ($OD_{620} \cong 0.6$) of NT-1(*traR, tra::lacZ749*) (Piper et al, 1993). After 22 h incubation at 25° C, cells were collected by centrifugation and assayed for β-galactosidase activity (Stachel et al., 1985).

Probe-EI Mass Spectrometry (EI-MS)

In all cases, the probe temperature was programmed from 50° C to 400° C at 100° C/min. Electron energy and emission current were 70 eV and 200 μA respectively. The reagent gas was isobutane. A Finnigan Mat TSQ 70 mass spectrometer was used for all mass spectrometric analyses.

Probe-CI Mass Spectrometry (CI-MS)

The probe temperature was programmed from 50° C to 400° C at 100° C/min, the source temperature was 150° C, and isobutane was used as the reagent gas.

MS/MS Mass Spectrometry

Daughter ion and parent ion scan modes were used in the study.

NMR Spectrometry

NMR spectra were recorded at 300 MHz with a Bruker HXS-360 NMR spectrometer at the University of Adelaide. Solvent used for NMR analysis was $CDCl_3$. Chemical shifts have been quoted in parts per million (ppm) downfield from tetramethylsilane. Multiplicities have been abbreviated to s, singlet; d, doublet; t, triplet; m, multiplet; br, broad.

Infrared Spectra (IR)

Infrared spectra of all chemical samples (~ 0.5%, KCl discs) were recorded on a Perkin Elmer 983 G infrared spectrometer.

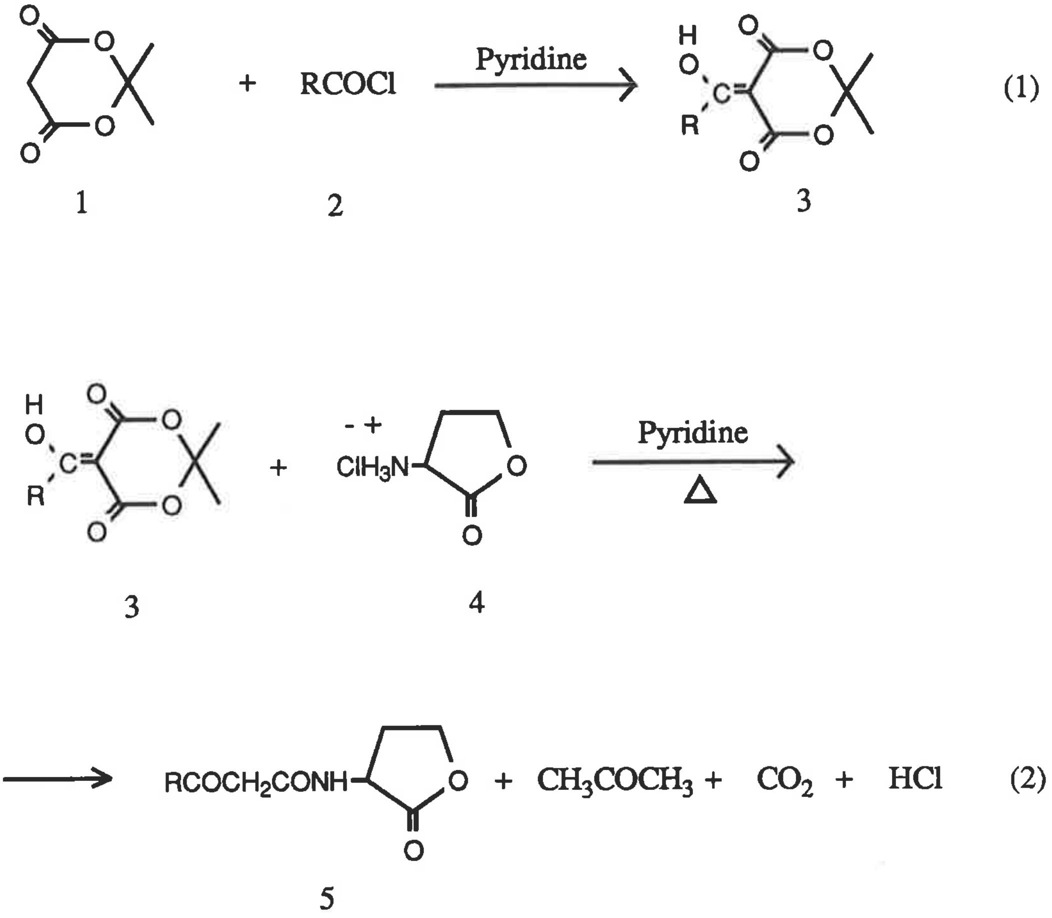
Amino Acid Analysis

A 200 µg sample of natural CF1 was hydrolysed in 5.7 M HCl for 16 h at 110° C, dried in a stream of nitrogen, and separated by high voltage paper electrophoresis in a formic acid-acetic acid (1.0 M HAc/0.75M HCOOH, pH1.75) system. Amino acids were detected by ninhydrin reaction (Vogel, 1969).

Synthesis Of CF And Its Derivatives

Synthetic CF1 (*N*-ß-oxo-octanoyl-L-homoserine lactone) was obtained in 49% yield in a one step procedure using L-homoserine lactone HCl (1m mole) in pyridine (10ml) at 130° for 45 minutes with the hexanoyl Meldrum's acid intermediate of Oikawa (Oikawa et al, 1978) (1.5 mmole). Cognate preparations of ß-oxo-hexanoyl and ß-oxo-decanoyl amides proceeded smoothly with the appropriate intermediate (Fig 4.1, compound 3). Saturated acyl amides were prepared from the L-homoserine lactone HCl and corresponding acyl chloride in pyridine under the same conditions. The methyl ester of CF1 was prepared by heating (50° C) the methanol solution of CF1 for 45 min in the presence of a small amount of DOWEX $50H^+$.

N-acyl amides were monitored in the preparation and purification processes



N-ß-oxo-octanoyl-L-homoserine lactone (CF1): R = $(CH_2)_4CH_3$

N-ß-oxo-hexanoyl-L-homoserine lactone (Autoinducer): R = $(CH_2)_2CH_3$

N-ß-oxo-decanoyl-L-homoserine lactone: R = $(CH_2)_6CH_3$

Fig. 4.1 Synthetic scheme of unsaturated acyl amides of homoserine lactone by silica plate TLC using ethyl acetate as solvent. Detection with I_2 vapour yielded a characteristic colour reaction: first white (compared with the background) then becoming dark yellow. This sequence was observed for all N-acyl amides tested.

Except for the methyl ester of CF1 which was purified by silica plate TLC, synthetic CF and its derivatives were purified by the following procedures. Pyridine in the reaction mixture was removed by adding 3 x 20 ml of toluene and evaporating at 45° C in a rotary vacuum evaporator. The residue was extracted with hot water (40° C, 5 x 20 ml). Crystallisation was achieved for saturated N-acyl amides when concentrated water solutions were left overnight at room temperature. N-ß-oxo-acyl amides were evaporated to dryness, redissolved in 1 ml of 30% methanol and purified by C18 reverse phase flash column chromatography eluted with 30% methanol.

Induction Of Ti Plasmid Conjugal Transfer By Conjugation Factor

Conjugation factor solutions were added to the octopine (200mg/litre) induction medium (Chapter 2) and then inoculated with a 1:40 dilution of a late log phase culture (OD620 ≈ 0.6) of Tra^-Cfs^- donor strain M103. Then 150 µl of a buffer saline-washed late log phase culture of the recipient strain K749 was spreaded onto each selective plate (Chapter 2). After 24 h incubation at 25° C, the drop mating method described in Chapter 2 was used to assay for Ti plasmid conjugal transfer efficiency.

RESULTS AND DISCUSSION

4.1 Screening For A High Yielding CF Strain

Signal molecules are usually present at very low levels in organisms, and the efficiency of CF production by octopine strains is variable (Zhang and Kerr, 1991; Chapter 3). Therefore selection of a high yielding strain of CF was necessary for purification and subsequent characterisation. Because wild type octopine strains cannot produce CF in liquid cultures (Chapter 3), only Tra^{c} and Tra^{ci} strains were screened. Among them, strain K794 gave the best yield of CF when grown in Petit's liquid induction medium (Petit and Tempé, 1978) supplied with proline (4 g/litre) as nitrogen source and mannitol (10 g/Litre) as carbon source (Table 4.1).

TABLE 4.1 CF production efficiency of some *Agrobacterium* octopine strains

Strains	CF activity at different dilutions			
	10^{0}	10^{-1}	10^{-2}	10^{-3}
K608	+	+	-	-
K794	+	+	+	+
K804	+	+	-	-
K588	+	+	+	-

4.2 Extraction Of CF By Organic Solvents

One of the first steps necessary in the purification of a compound from a complex mixture such as a bacterial culture solution is to remove the bulk of the salts and nutrients. In this particular instance, the partition properties of the biological active components permitted extraction by organic solvents.

Solvents of different polarity were selected to determine the efficiency of extraction. A culture filtrate of K794 (2 ml) was extracted with the same volume of organic solvent by shaking in a 10 ml disposable centrifuge tube for 5 min. The organic and aqueous phases were separated after centrifugation (5000 rpm x 2 min). Table 4.2 shows that chloroform and ethyl acetate gave the best extraction of CF from culture filtrates. The cyclohexane data also indicate that CF is amphiphilic. The two phases of the otherwise miscible solvent acetone were due to salt content of the medium.

TABLE 4.2 Organic solvent extraction of CF from culture filtrates

Extracting solvents	Solution phases	CF activity at different dilutions		
		10^0	10^{-1}	10^{-2}
Chloroform	Organic	+	+	+
	Aqueous	-	-	-
Ethyl acetate	Organic	+	+	+
	Aqueous	-	-	-
Acetone	Organic	+	+	-
	Aqueous	+	-	-
Cyclohexane	Organic	-	-	-
	Aqueous	+	+	+

4.3 High Voltage Paper Electrophoresis Of CF

High-voltage paper electrophoresis was used to determined whether CF is charged or not with a view to providing information to design a suitable chromatographic method for purification.

Table 4.3 shows that CF always stays at the origin after electrophoresis in different buffer systems, indicating that CF contains no readily ionisable groups, and hence ion-exchange chromatographic methods would be unsuitable for the purification of CF.

TABLE 4.3 Mobility of CF after high voltage paper electrophoresis in different buffer systems

Buffer system	pH	Mobility
Tris-citrate	7.5	0.0
Citrate	6.0	0.0
Formic acid-acetic acid	1.75	0.0

4.4 Chromatographic Separation of CF

Silica, C18 reverse phase silica and cellulose were examined as potential stationary phases for the chromatographic purification of CF. To provide parameters for choosing an efficient large scale chromatographic method for purifying CF, paper chromatography and thin layer chromatographic Rf values were obtained.

Table 4.4 shows the Rf values observed for 6 different solvent systems in paper chromatography. It can be seen that Rf values of CF remain very high in all solvent systems, suggesting that cellulose is not a suitable stationary phase for CF purification.

Table 4.5 and Table 4.6 show Rf values of CF in silica gel and C18 reverse phase thin layer chromatography. The Rf values varied widely in different solvent systems, indicating that CF can be separated by using silica and C18 reverse phase columns. The data in Table 4.5 also suggest that CF contains two components as indicated by two biologically active spots following TLC on silica gel in chloroform/water (70:30) and in chloroform/methanol/water (70: 25:5) solvent systems.

TABLE 4.4 Rf values of CF biological activity after paper chromatography in a number of solvent systems

Solvent system	Rf
Butanol: water: acetic acid=75:20:5	0.92
Chloroform:methanol:water=70:25:5	0.98
Ethyl acetate:pyridine:water=80:20:10	0.99
Isopropanol:water=80:20	0.88
Isopropanol:water:acetic acid=50:50:1	0.97
Isopropanol:water:acetic acid=90:5:5	0.93

4.5 Large Scale Purification of CF

Large scale purification of CF was carried out using the information provided by the preliminary experiments. A culture filtrate of strain K794 was extracted with ethyl acetate, and then the concentrated CF extracts were purified by silica gel column and C18 reverse phase column flash chromatography. Evaporation of the active fractions gave a yellowish white solid (3 mg/L culture). Further purification by C_{18} reverse phase HPLC gave two separate biologically active components, labelled CF1(retention time 13.38 min.) and CF2 (retention time 11.4 min). It was noted that these two fractions were almost identical in their fraction peaks as indicated by the absorption at 215 nm and in their biological activity.

TABLE 4.5 Rf values of CF biological activity after silica gel thin layer chromatography in five solvent systems

Solvent systems	Rf
Chloroform:water=70:30	0.82; 0.73
Chloroform:methanol=90:10	0.40
Chloroform:methanol:water=70:25:5	0.78; 0.71
Butanol	0.34
Ethyl acetate	0.51

TABLE 4.6 Rf values of CF biological activity after C18 reverse phase thin layer chromatography in three solvent systems

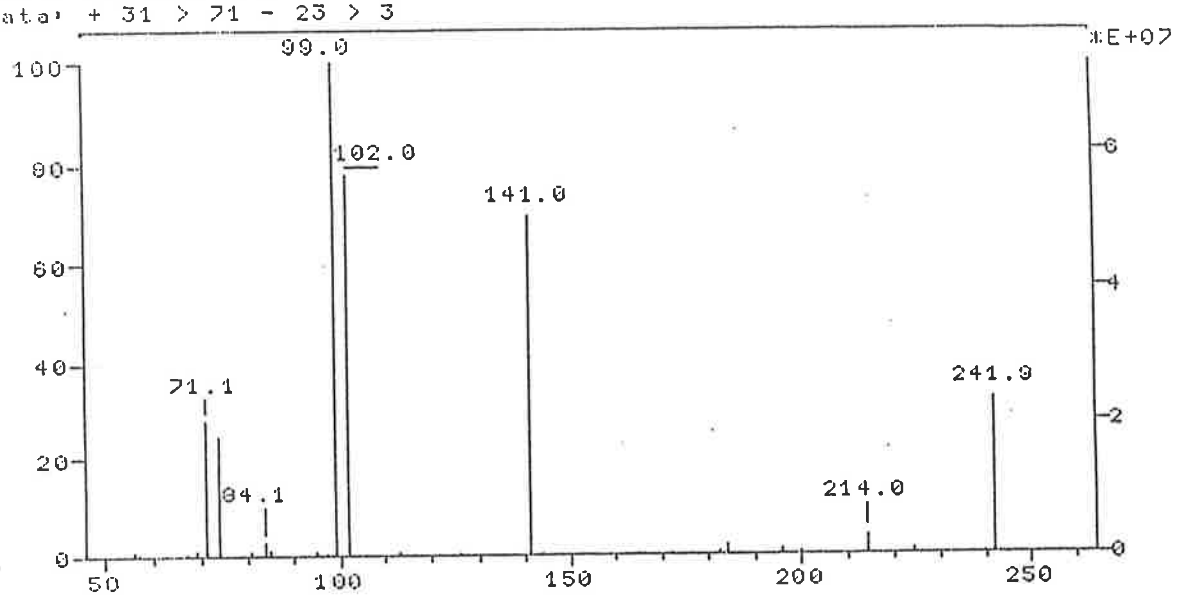
Solvent system	Rf
Acetonitril : water = 80 : 20	0.91
Acetonitril	0.89
Methanol : water= 70 : 30	0.47

4.6 Characterisation Of CF1 And CF2

Purification of CF by HPLC yielded two active components, CF1 and CF2. Examination of these fractions by chemical ionisation, quadrupole mass spectrometry showed strong quasi molecular (M+H) ions, with m/z 242 for CF1 (Fig 4.2); and the less homogeneous CF2 fraction showed ions at m/z 274, 214 and 200 (Fig 4.3, 4.4, 4.5). All of these even M+H ions were consistent with the presence of nitrogenous components. For CF1 this was confirmed by infrared spectrometry, which showed amide I and II carbonyl absorptions at 1646 and 1540 cm^{-1} as well as characteristic absorptions near 1780 cm^{-1} and 1714 cm^{-1} (Table 4.9) assignable to the presence of γ lactone and ketonic carbonyls (Eberhard et al, 1981). Acid hydrolysis yielded an amino acid which co-migrated with homoserine when separated by high voltage paper electrophoresis (Fig.4.6). Daughter ion tandem mass spectrometry of the M+H ions at 242m/z in CF1, and M+H ions at 274, 214 and 200 in CF2, showed related fragmentations after helium collisional activation. Except for the 214 parent ion which showed a daughter ion at 113 instead of 99 as well as ions at 102 and 141, each daughter ion set from the parent ions at 242, 274 and 200m/z, displayed prominent 99, 102 and

Fig. 4.2 DI-CI mass spectra of natural CF1 (Top) and synthetic CF1: N-ß-oxo-octanoyl-L-homoserine lactone (Bottom).

```
SPEC: YH92AU21H ver 1 on UIC 002002  21-AUG-92 DERIVED SPECTRUM     9
Samp: CF1                                      Start : 16:41:33    10
Comm: DI-CI(ISOBUTANE) 50(0.25)-400,@100C/MIN
Mode: CI +DAU 242.0 @ -30eV LMR SYNTH GAS UP LR
Oper: YOJI                                     Inlet :
Base: 99.0                 Inten : 72581200    Masses: 50 > 260
Norm: 99.0                 RIC   : 254276432   # peaks: 241
Peak: 1000.00 mmu
Data: + 31 > 71 - 23 > 3
```



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SPEC2: _
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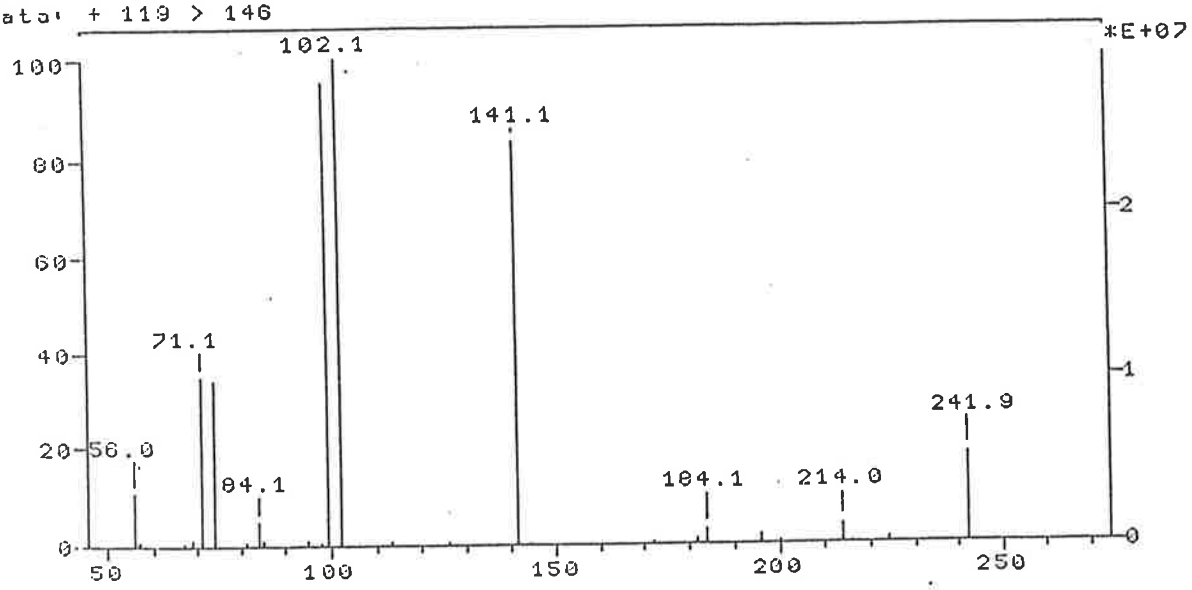
SPEC: YH92AU07A ver 1 on UIC 7 11      7-AUG-92 DERIVED SPECTRUM     9
Samp: SCF1                                      Start : 08:11:21    10
Comm: DI-CI(ISOBUTANE)
Mode: CI +DAU 242.0 @ -22eV LMR SYNTH GAS UP LR
Oper: YOJI                                      Inlet :
Base: 102.1                Inten : 29163722     Masses: 50 > 270
Norm: 102.1                RIC   : 110163824    # peaks: 203
Peak: 1000.00 mmu
Data: + 119 > 146
```



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SPEC2: _
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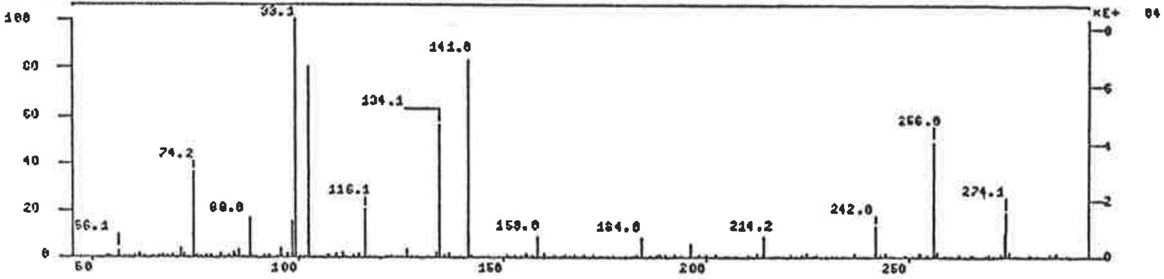
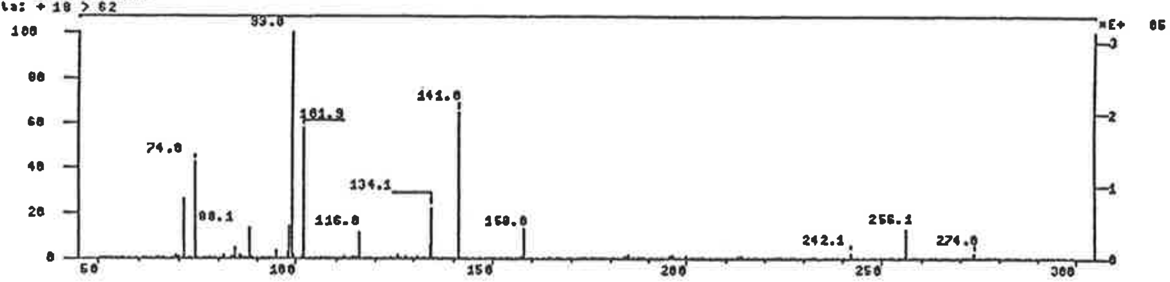
Fig. 4.3 DI-CI mass spectra of natural m/z=274 (M+H) component in CF2 fraction (Top) and synthetic methyl ester of CF1 (Bottom).

```
SPEC: VH92AU216 ver 1 on UIC 002002  21-AUG-92 DERIVED SPECTRUM      9
Samp: CF2                                     Start : 16:22:36      10
Comm: DI-CI(ISOBUTANE) 50(0.25)-400,0100C/MIN
Mode: CI +DAU 274.0 @ -30eV LHR SYNTH GAS UP LR
Oper: YOJI                                    Inlet :
Base: 93.1              Inten : 82928         Masses: 50 > 290
Norm: 93.1              RIC   : 630540        # peaks: 196
Peak: 1000.00 mmu
Data: + 14 > 54 - 54 > 34
```



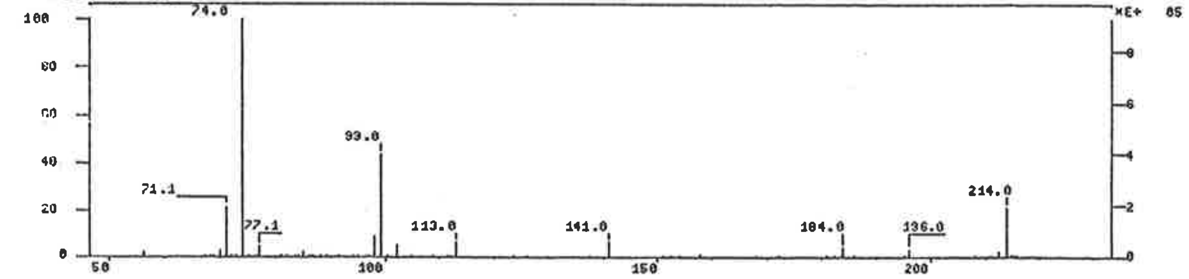
```
SPEC: VH92SP16A ver 3 on UIC 007011  16-SEP-92 DERIVED SPECTRUM      9
Samp: MEESTER OF SCF1                         Start : 15:23:47      10
Comm: CI(ISOBUTANE)-MS/MS
Mode: CI +DAU 274.1 @ -30eV LHR SYNTH GAS UP LR
Oper: YOJI                                    Inlet :
Base: 93.0              Inten : 313141        Masses: 50 > 300
Norm: 93.0              RIC   : 1405247       # peaks: 340
Peak: 1000.00 mmu
Fltr: 1P&2P&LR&DA&CI of M/Z 273.8 > 274.6
Data: + 18 > 62
```



SPEC2:

Fig. 4.4 DI-CI mass spectra of natural m/z=214 (M+H) component in CF2 fraction (Top) and synthetic autoinducer of luminescence in *Vibrio fischeri*: N-ß-oxo-hexanoyl-L-homoserine lactone (Bottom).

```
SPEC: VH32AU21H ver 1 on UIC 002002  21-AUG-92 DERIVED SPECTRUM      9
Samp: CF2                                      Start : 16:41:33     10
Comm: DI-CI(ISOBUTANE) 50(0.25)-400,@100C/MIN
Mode: CI +DAU 214.0 @ -30eV LMR SYNTH GAS UP LR
Oper: YOJI                                     Inlet :
Base: 74.0                 Inten : 922130      Masses: 50 > 230
Norm: 74.0                 RIC   : 2217666     # peaks: 196
Peak: 1000.00 mmu
Data: + 26 > 54 - 22 > 2
```



```
SPEC: VH32AU21J ver 1 on UIC 002002  21-AUG-92 DERIVED SPECTRUM      9
Samp: SAI                                      Start : 17:16:41     10
Comm: DI-CI(ISOBUTANE) 50(0.25)-400,@100C/MIN
Mode: CI +DAU 214.0 @ -30eV LMR SYNTH GAS UP LR
Oper: YOJI                                     Inlet :
Base: 71.0                 Inten : 107063416   Masses: 50 > 230
Norm: 71.0                 RIC   : 368232304   # peaks: 219
Peak: 1000.00 mmu
Data: + 33 > 65 - 29 > 0
```

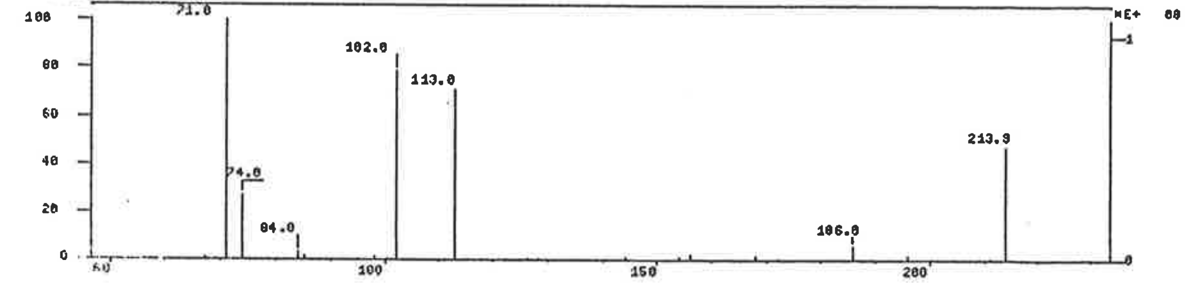
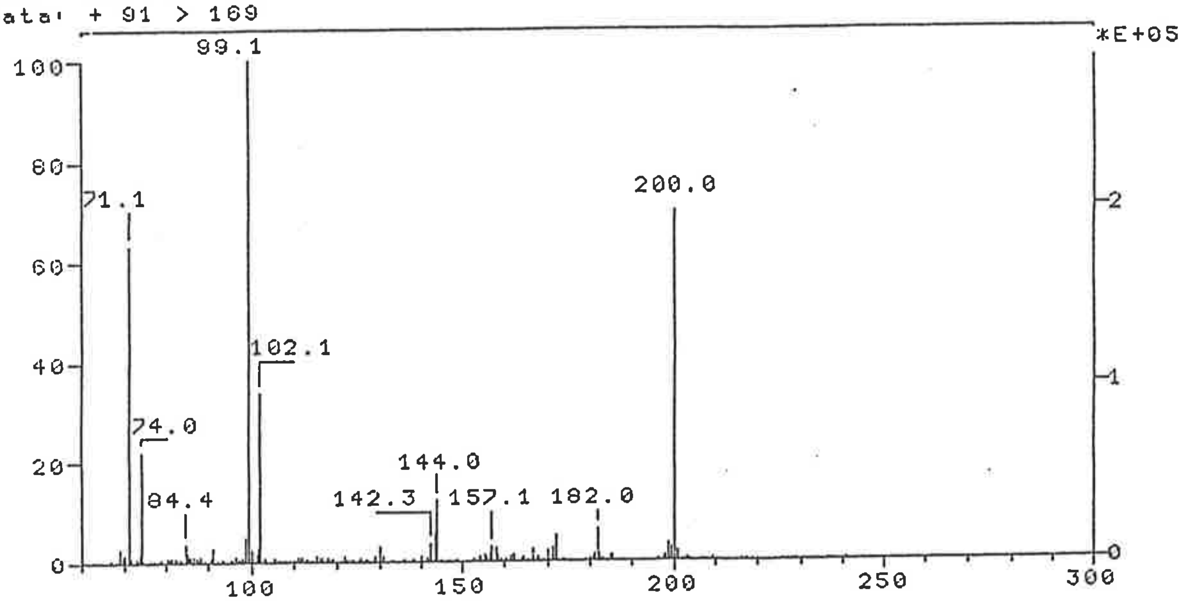
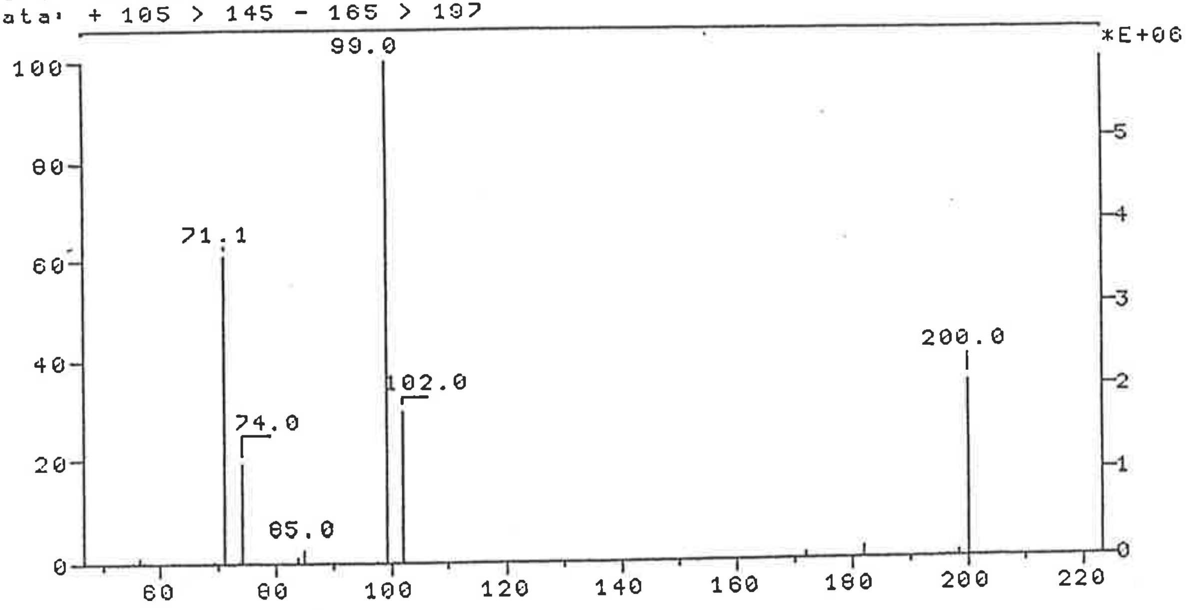


Fig. 4.5 DI-CI mass spectra of natural m/z=200 (M+H) component in CF2 fraction (Top) and synthetic N-hexanoyl-L-homoserine lactone (Bottom).

```
SPEC: YH92AU07D ver 1 on UIC 7 11      7-AUG-92 DERIVED SPECTRUM      9
Samp: CF2 MS/MS                        Start : 09:09:46              10
Comm: DI-CI(ISOBUTANE)
Mode: CI +DAU 200.0 @ -22eV LMR SYNTH GAS UP LR
Oper: YOJI                                      Inlet :
Base: 99.1            Inten : 282265            Masses: 30 > 250
Norm: 99.1            RIC   : 1375487           # peaks: 233
Peak: 1000.00 mmu
Data: + 91 > 169
```



```
SPEC: YH92AU21I ver 1 on UIC 002002   21-AUG-92 DERIVED SPECTRUM      9
Samp: SHA  CI-MS/MS                    Start : 16:57:55              10
Comm: DI-CI(ISOBUTANE) 50(0.25)-400,@100C/MIN
Mode: CI +DAU 200.0 @ -30eV LMR SYNTH GAS UP LR
Oper: YOJI                                      Inlet :
Base: 99.0            Inten : 5927642           Masses: 50 > 220
Norm: 99.0            RIC   : 15200424          # peaks: 164
Peak: 1000.00 mmu
Data: + 105 > 145 - 165 > 197
```



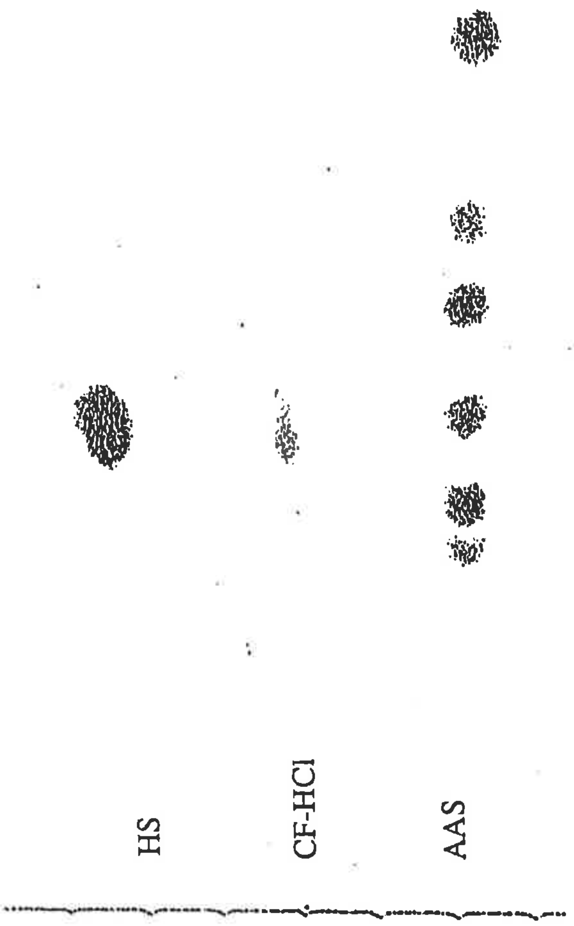


Fig. 4.6 High voltage paper electrophoresis (pH 1.75, 3000V/15min) detection of the homoserine component in CF1 after acid hydrolysis. HS: homoserine standard; CF1-HCl: CF1 acid hydrolysis sample: AAS: amino acids mixture standard (In decreasing mobility order: arginine; glycine; .alanine; serine + valine + isoleucine; glutamic acid, aspartic acid).

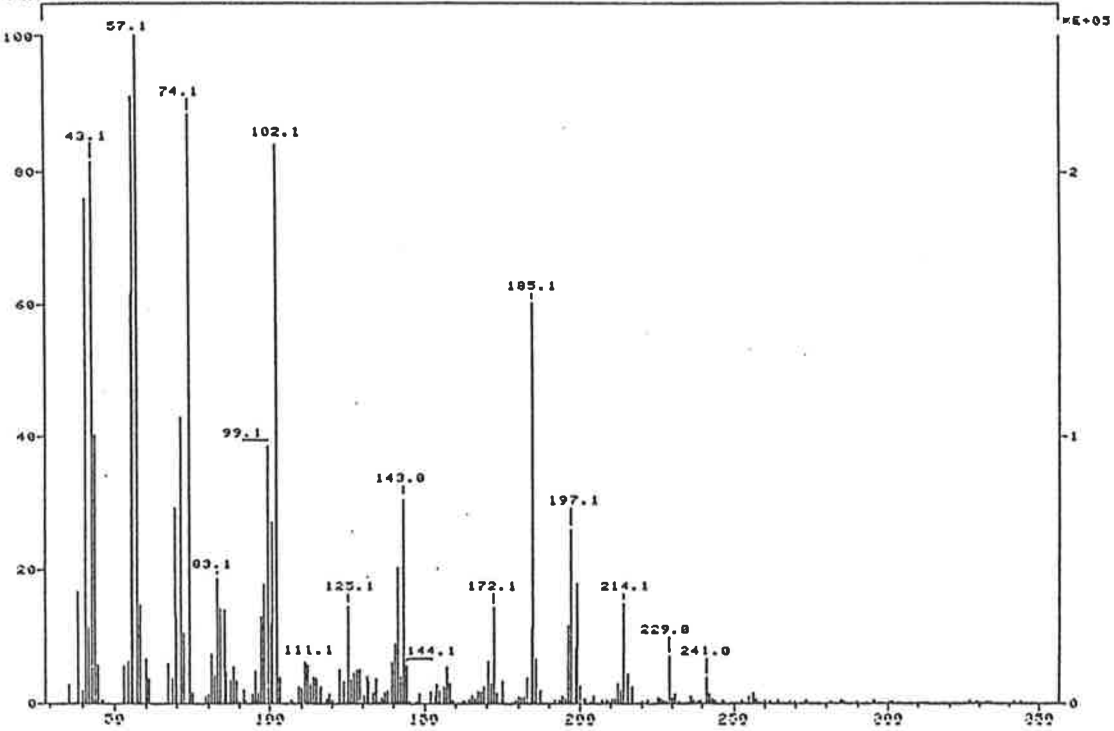
141 m/z ions. Comparison of the corresponding electron impact data with that reported by Eberhard et al (1981) suggested a closely related set of homoserine lactone derivatives.

The direct-electron impact mass plot of natural CF1 is shown in Fig. 4.7. Although it is not a precise mass measurement, the relevant information from IR and NMR makes the assignment possible. The corresponding assigned elemental compositions are presented in Table 4.8. The molecular radical ion of CF1 had m/z=241.1, consistent with the formula $C_{12}H_{19}O_4N$ (calculated m/z=241.13). The molecular ion displayed losses corresponding to C_3H_7, C_4H_9, C_5H_{11}, $C_6H_{11}O$ and $C_8H_{13}O_2$ fragments, suggesting the presence of a ß-oxo-octanoyl group. The rearrangement fragment $C_8H_{15}O_2N$ indicates the attachment of the ß-oxo-octanoyl group to an amino moiety. A prominent fragment $C_4H_8O_2N$ is consistent with a loss of $C_8H_{12}O_2$, the ß-oxo-octanoyl group from the molecular ion.

The COSY spectrum of the natural CF1 (Fig. 4.8) shows two types of ^1H-^1H coupling relationships. The coupling relationship and chemical shifts of H5 (0.82 ppm, triplet, H=3)/H4 (1.27 ppm, multiplets, H=2)/H3, H2 (1.56 ppm, multiplets, H=4)/H1 (2.52 ppm, triplets, H=2) indicate the presence of a pentyl group which is likely to be attached to a carbonyl functionality. Measurement of the integration at the signal 1.56 ppm shows the number of hydrogens in that peak is 4, therefore, this signal is assigned to H3 and H2. The coupling of H7(7.72 ppm, doublet and broad, H=1)/H11 (4.47 ppm, trip-duplets, H=1)/H10 (2.20 and 2.75 ppm, multiplets, H=2)/H9 (4.26 and 4.58 ppm, multiplets, H=2) corresponds a -NH-CH-CH_2-CH_2- moiety. The broad doublet signal at 7.72 ppm (J=6.8 HZ) is typical of an NH in a polypeptide. The tri-doublets signal at 4.47 ppm ($J\alpha\beta$ =9.1 Hz, $J\alpha\nu$ =4.55 Hz) is also typical of a α-CH in a polypeptide. The chemical shift pattern of H11/H10/H9 is consistent with a five-membered lactone ring with H9 closing to an electron negative heteroatom, i.e. oxygen (Williams and Fleming, 1989). The signal at 7.25 ppm is due to the chloroform residue in the $CDCl_3$ solvent (Williams and Fleming, 1989). The signal at 3.47 ppm might be due to the -CH_2- between carbonyl and carboxyamide groups. The weak signal at 3.78 ppm was thought

Fig. 4.7 DIRECT-EI mass spectra of natural CF1 (Top) and Synthetic CF1 (Bottom).

```
SPEC: YH92JN30B ver 3 on UIC 7 11     30-JUN-92 DERIVED SPECTRUM      9
Samp: CF1                                       Start : 17:17:08      10
Comm: DIRECT-EI(70EV)
Mode: EI +Q3MS LMR UP LR
Oper: YOJI                                      Inlet :
Base: 57.1               Inten : 249842         Masses: 35 > 350
Norm: 57.1               RIC   : 3688111        # peaks: 264
Peak: 1000.00 mmu
Data: + 79 > 90 - 44 > 55
```



```
SPEC: YH92AU21J ver 3 on UIC 002002  21-AUG-92 DERIVED SPECTRUM      9
Samp: SCF1 EI                                  Start : 18:13:21     10
Comm: DI-EI
Mode: EI +Q3MS LMR UP LR
Oper: YOJI                                     Inlet :
Base: 185.0              Inten : 2345356       Masses: 35 > 500
Norm: 185.0              RIC   : 24916874      # peaks: 393
Peak: 1000.00 mmu
Data: + 48 > 68 - 68 > 77 - 42 > 32
```

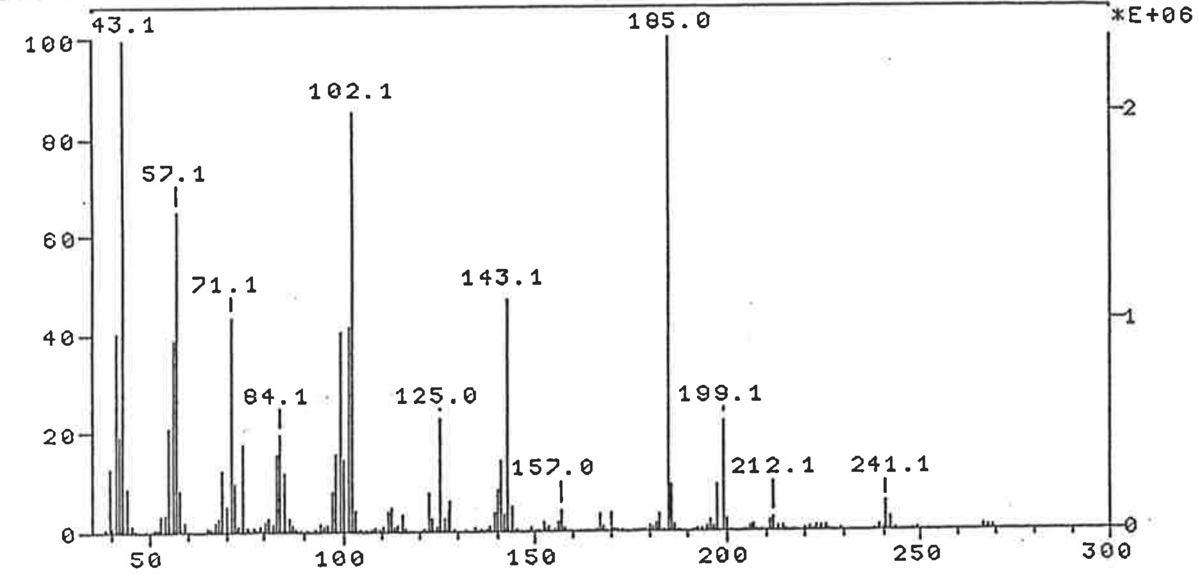
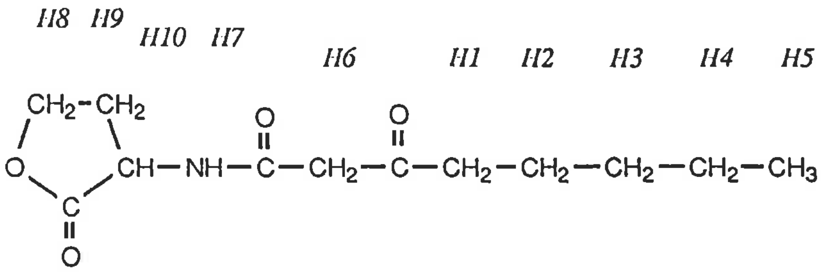


Fig. 4.8 COSY NMR spectrum of natural CF1.


H8 H9
H10 H7
H6
H1
H2
H3
H4
H5
CH2-CH2
O
CH—NH—C—CH2—C—CH2—CH2—CH2—CH2—CH3
C
O
O
O


H7
H10
H8
H8
H6
H9
H1
H9
H3
H2
H4
H5
7.0
6.0
5.0
4.0
3.0
2.0
1.0
PPM

to be due to a contaminant which was not present in the ^{1}H NMR spectrum of natural CF1 (Table 4.8).

These results strongly suggest that CF1 is *N*-(ß-oxo-octan-1-oyl)-L-homoserine lactone (Fig. 4.9) which was confirmed by IR spectrum data (Table 4.9), mass spectrum data (Fig. 4.2, Fig. 4.7), ^{1}H NMR (Table 4.8) and the biological activity (Fig. 4.10) of synthetic CF1.

TABLE 4.7 Mass spectrum of natural CF1

m/z	Elemental composition	m/z by calculation
43.1	C_3H_7	43.05
57.1	C_4H_9	57.07
71.1	C_5H_{11}	71.09
99.1	$C_6H_{11}O$	99.08
102.1	$C_4H_8O_2N$	102.06
141.0	$C_8H_{13}O_2$	141.09
143.0	$C_6H_9O_3N$	143.06
157.0	$C_8H_{15}O_2N$	157.11
172.1	$C_7H_{10}O_4N$	172.06
185.1	$C_8H_{11}O_4N$	185.07
197.1	$C_9H_{11}O_4N$	197.07
214.1	$C_{10}H_{16}O_4N$	214.11
241.1	$C_{12}H_{19}O_4N$	241.13

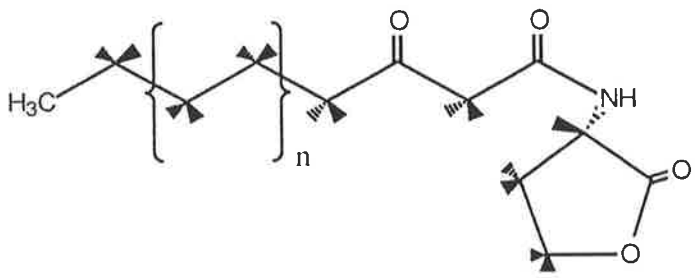


Fig.4.9 Structural formulae for *N*-β-oxo-acyl- amides of L-homoserine lactone, n =0, 1, 2 correspond to the *N*-β-oxo-hexanoyl, -octanoyl and -decanoyl amides.

Synthesis of the known luciferase autoinducer β-oxo-hexanoyl homoserine lactone of *V. fischeri* reported by Eberhard et al (1981) (Fig 4.9 structure n=0), M+H 214m/z, confirmed its relatedness to, but non-identity with the tandem ion mass spectrometry daughter ion spectra from the 214 m/z parent ion in the CF2 fraction (Fig. 4.4).

TABLE 4.8 ^{1}H NMR spectral data of natural CF1 and synthetic CF1*

	^{1}H NMR
1	2.52, t
2	1.56, m
3	1.56, m
4	1.27, m
5	0.82, t
6	3.47, s
7	7.72, br, d
8	4.46-4.58, m
9	2.20-2.75, m
10	4.47, m

* The differences between the data of CF1 and those of synthetic CF1 are smaller than 0.005ppm; the data of natural CF1 is listed in the table.

The tandem mass spectrometric fragments of the M+H ion of m/z 200 present in the CF2 fraction suggested it was the fully reduced hexanoyl amide of homoserine lactone and the daughter ion fragmentation patterns were indistinguishable from a synthetic sample of the hexanoyl amide (Fig. 4.5).

The close relationship of the daughter ions from the 274 parent ion to those of CF1 m/z 242 and in particular the presence of a strong fragment ion at 134 instead of the ion corresponding to a protonated homoserine lactone at 102, suggested that CF2 fraction contained the methyl ester of the conjugation factor derived as a methanolic-solvent transesterification artifact. Therefore, the methyl ester of CF1 was synthesized. Fig 4.3 shows that its CI daughter ion mass spectrum is the same as that of the M+H ion of m/z 274 present in the CF2 fraction which was further confirmed by its chromatographic behaviour on TLC and HPLC.

TABLE 4.9 IR spectra of CF1 and its derivatives

Compound*	Main bands (cm^{-1})					
Natural CF1	1780	1714	1646	1540	1173	1023
CF1	1780	1712	1644	1538	1172	1022
Autoinducer	1779	1715	1646	1547	1174	1021
N-ß-oxo-decanoyl-l-homoserine lactone	1782	1712	1645	1540	1172	1022
N-hexanoyl-l-homoserine lactone	1775	-	1642	1545	1173	1014
N-octanoyl-l-homoserine lactone	1775	-	1642	1546	1173	1013

* Except for natural CF1, all compounds are synthetic.

4.7 Biological Activity

The natural and synthetic compounds were tested for biological activity. Fig. 4.10 shows *tra* gene activity induced by natural CF1 from *A. tumefaciens* and by synthetic *N*-(β-oxo-octanoyl)-, methyl ester of CF1 and *N*-hexanoyl amides of L-homoserine lactone. The synthetic *N*-β-oxo-octanoyl-L-homoserine lactone shows identity in gene induction activity with the natural CF1 and accounts fully for the observed activity of fraction CF1. Although masses corresponding to the *N*-hexanoyl amide were detected by mass spectrometry in the biologically active CF2 fraction from the HPLC fractionation, the synthetic product had insufficient activity on a molar basis to account for the activity of this fraction (ref to section 4.5). The biological activity of methyl ester of CF1 are almost the same as that of natural CF1 and this can well explain the biological activity observed for CF2 fraction (Section 4.5). This similarity in activity may be due to intracellular lactonisation of CF1 methyl ester in the bacterium.

4.8 Specificity Of Conjugation Factor In tra Gene Induction

The fact that N-hexanoyl amide of homoserine exhibited very low biological activity indicates that the length and the nature of the lipophilic acyl chain of homoserine amide could be important in the *tra* gene inducing activity (Fig 4.10). A series of CF1 derivatives were synthesized and their biological activities determined. The remarkable feature of Fig. 4.11 is that extension (Fig 4.9, n = 2) or diminution (Fig 4.9, n = 0) of the chain length or complete conversion of the beta keto groups to methylene groups (Fig 4.9, n = 0 and n = 1) was sufficient to reduce the inducing activity by four orders of magnitude or more compared to that of the β-oxo-octanoyl derivative (Fig 4.9, n = 1). A similar specificity was reported for analogues of the autoinducer of bioluminescence in *V. fischeri* (Eberhard et al., 1986).

The facts that *N*-(β-oxo-hexanoyl)-homoserine lactone is the autoinducer for luminescence activity in *V. fischeri* (Eberhard et al, 1981) and for antibiotic synthesis in *Erwinia* (Bainton et al., 1992), that *N*-β-(hydroxy-butanoyl)-L-homoserine lactone is the

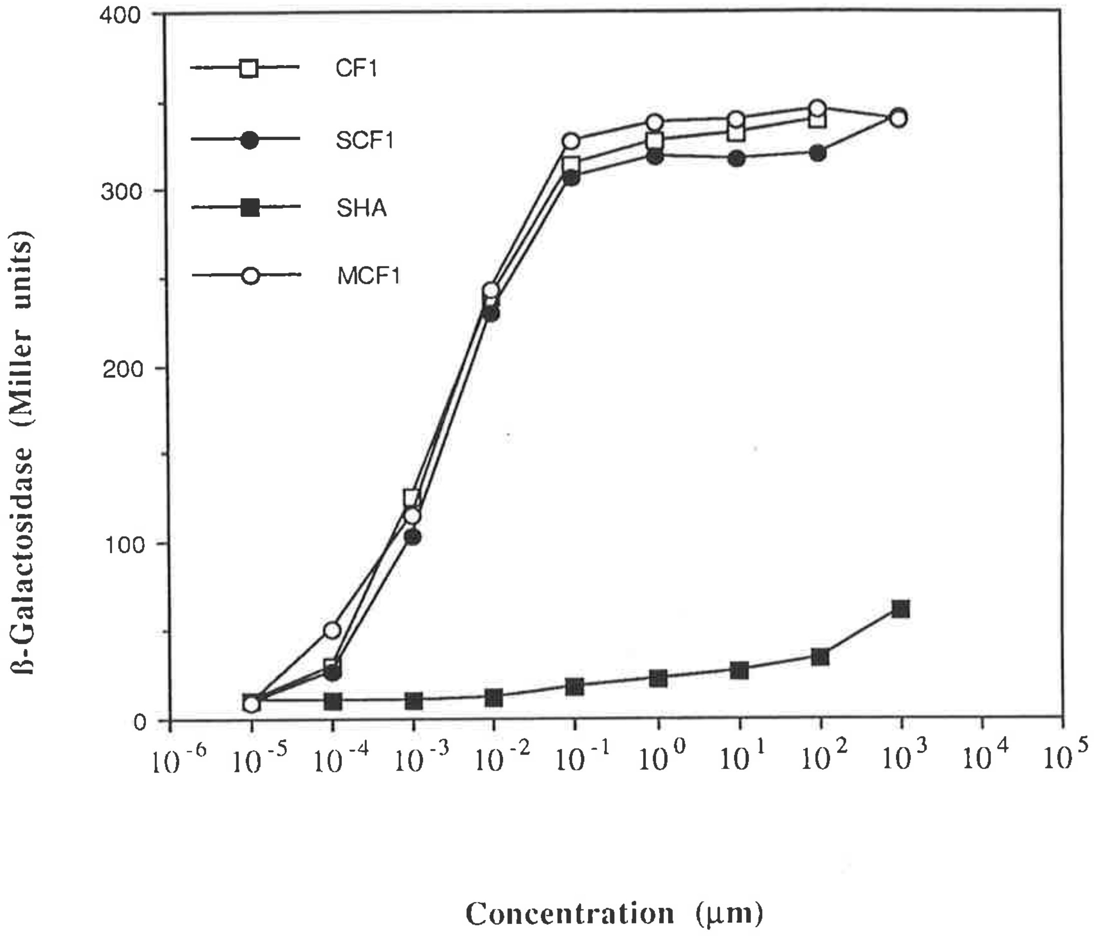


Fig.4.10 Induction of *tra* gene activity by Natural CF1(CF1), synthetic *N*-ß-oxo-octanoyl- (SCF1), synthetic methyl ester of N-ß-oxo-octanoyl- (MCF1), and synthetic *N*-hexanoyl-(SHA) amides of L-homoserine lactone.

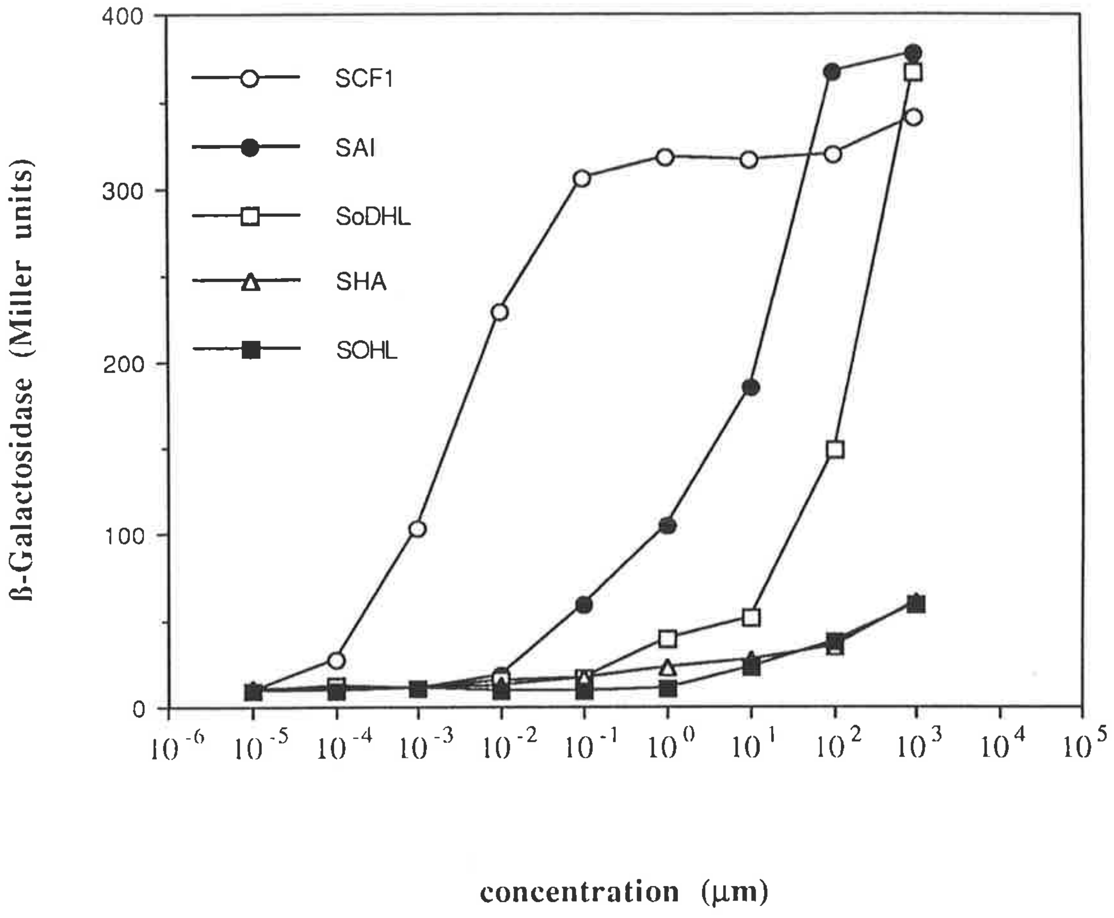


Fig.4.11 Selective induction of *tra* gene activity by CF1 and its derivatives. Abbreviations: SCF1 (synthetic CF1), SAI (synthetic N-ß-oxo-hexanoyl-L-homoserine lactone, the autoinducer of luminescence of *V. fischeri*), SoDHL (synthetic N-ß-oxo-decanoyl-L-homoserine lactone), SHA (synthetic *N*-hexanoyl- L-homoserine lactone), and SOHL (synthetic N-octanoyl-L-homoserine lactone).

autoinducer for luminescence in *V. harveyi* (Cao and Meighen, 1989) and that *N*-(β-oxo-octanoyl)-L-homoserine lactone is the autoinducer for conjugation in *A. tumefaciens* suggest that *N*-acyl homoserine lactones may be a widely conserved signal for gene regulation.

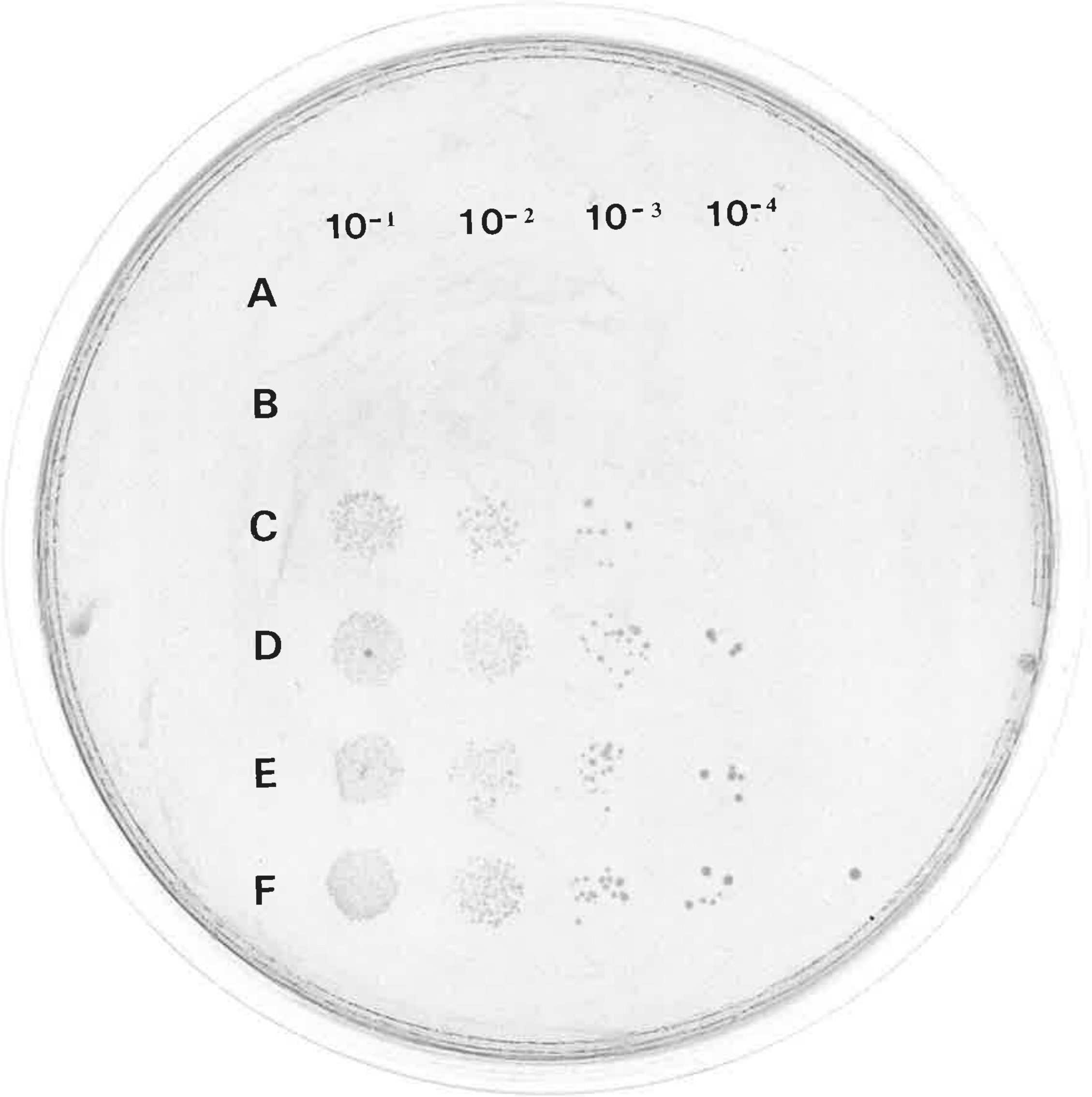
4.9 Induction of Ti plasmid Conjugal Transfer

An experiment was carried out to determine the relationship of conjugation factor concentration and Ti plasmid conjugal transfer efficiency in *Agrobacterium*. The donor strain M103 is a Cfs$^-$ mutant mediated by Tn*5* insertional mutagenesis (Chapter 8). The donor strain was grown for 24 h in liquid octopine induction medium containing different concentrations of synthetic CF1 before mating with the recipient strain K749. Fig.4.12 shows that the induction of Ti plasmid conjugal transfer by conjugation factor is concentration dependent. No transconjugants (Ti plasmid transfer efficiency $< 1 \times 10^{-8}$) were observed when CF1 concentration was 1 nM. A clear increase in Ti plasmid conjugal transfer efficiency of strain M103 (8.6×10^{-4}) was observed when CF1 concentration increased to 10 nM. The maximal induction (Ti plasmid transfer efficiency $= 3.4 \times 10^{-3}$) was achieved when CF1 concentration was 100 nM. The data are very consistent with the results of *tra* gene induction (Fig. 4.10).

CONCLUSION

Conjugation factor was separated and purified from *Agrobacterium* culture filtrate by ethyl acetate extraction, silica and C18 reverse phase column flash chromatography, and C18 reverse phase HPLC. Conjugation factor produced by octopine strains of *Agrobacterium* was identified as *N*–β-oxo-octanoyl-L-homoserine lactone by nuclear magnetic resonance spectroscopy, infrared spectroscopy and, mass spectrometry. To verify this structure, CF was chemically synthesised by condensation

Fig.4.12 Ti plasmid conjugal transfer of *A. tumefaciens* strain M103 (Cfs$^-$) mutant following induction by external CF1. The recipient strain K749 was sprayed evenly onto the surface of the medium in this selective plate and incubated for 24 h before mating with donor strain M103 which was grown for 24 h in the presence of 0 nM (A), 1 nM (B), 10 nM (C), 100 nM (D), 1µM (E), and 10 µM (F) of synthetic CF1. A series dilutions (10^{-1} to 10^{-4}) of Strain M103 was prepared for mating and the dark spots in the plate show the growth of the corresponding transconjugants in the selective plate.


10^{-1}
10^{-2}
10^{-3}
10^{-4}
A
B
C
D
E
F

of β-oxo-hexanoyl meldrum acid with L-homoserine lactone chloride. The pure synthetic conjugation factor gave the characteristic IR, NMR, mass spectra and specified biological activity.

A series of CF derivatives were synthesised and tested for their biological activities. The results indicated that both the length and the nature (saturated or unsaturated) of the acyl chain were very important in specifying biological activities.

The conjugation factor is structurally very similar to *N*-ß-oxo-hexanoyl-L-homoserine lactone, the autoinducer of bioluminescence in a very dissimilar bacteria, *Vibrio fischeri* .

CHAPTER 5. OXYGEN RADICALS REGULATE CONJUGATION FACTOR BIOSYNTHESIS IN *AGROBACTERIUM*

INTRODUCTION

Although wild type octopine strains of *A. tumefaciens* produced conjugation factor (CF) in solid agar medium, no CF was detected in shaking liquid culture filtrates (Zhang and Kerr, 1991; Chapter 3). Clearly, the biosynthesis of CF is not only regulated by conjugal opines, but also by other factors. There were at least three obvious differences between solid agar plate cultures and shaking liquid cultures. They were (a) the presence or absence of agar; (b) shaking at 100 rpm or no shaking and (c) light conditions: continuous dark for the agar cultures and continuous light for the shaking cultures (Chapter 3). Preliminary experiments showed that CF synthesis inhibition resulted from shaking rather than from light or agar. However, in shake cultures bacteria not only receive constant mechanical impact but also an increased supply of oxygen. Therefore, experiments were designed, based on these differences, to pinpoint the real factor responsible for the regulation of CF biosynthesis in *A. tumefaciens* with the ultimate aim to have a better understanding of the regulation of biosynthesis of this interesting signal molecule and of Ti plasmid conjugal transfer.

MATERIALS AND METHODS

Bacterial Strains And Media

CF producing strain A6 and CF bioassay strain NT1 (traR, tra::lacZ749) have been described in Chapters 2 and 4 respectively. Bacterial maintenance medium and octopine induction medium were the same as described in Chapter 2. A concentration of 1 g/litre of octopine was used throughout the experiments described in this Chapter.

Chemicals And Enzyme

All chemicals used in the experiments described in this Chapter were from SIGMA or BDH. Catalase is the product of SIGMA.

Conjugation Factor Biosynthesis and Bioassay

To test for CF production by *A. tumefaciens* under different conditions, 40 μl of an overnight culture ($OD_{620} \approx 0.6$) of strain A6 was inoculated into 20 ml screw capped McArtney bottles which contained 5 ml (except when otherwise stated) of liquid minimal medium (Petit and Tempé, 1978) supplied with 1 g/litre of octopine and glucose as sole nitrogen and carbon sources. Bacteria were grown at 28° C on a rotary shaker (100 rpm, except when otherwise stated) for 24 h. CF in the culture filtrates was assayed by induction of a *tra::lacZ*749 reporter gene fusion in strain NT-1 (*traR*, *tra::lacZ*749). ß-galactosidase activity was expressed as Miller units (Miller, 1972). Bacterial culture filtrates were added in the proportion of 1:100 to minimal medium (Petit and Tempé, 1978) supplied with $(NH_4)_2SO_4$ (0.2%,w/v) and mannitol (0.2%,w/v) as nitrogen and carbon sources respectively, and then inoculated with a 1:40 dilution of a late log phase culture ($OD_{620} \approx 0.6$) of NT-1(*tra::lacZ749*). After 22 h incubation at 25° C, cells were collected by centrifugation and assayed for ß-galactosidase activity (Stachel et al, 1985). Each experiment was repeated at least three times.

Effect Of Oxygen Radicals Scavengers On CF Biosynthesis

Oxygen radical scavengers were added to the octopine induction medium before inoculating CF producing strain A6, and then incubated and assayed for CF activity in the culture filtrates as described above.

RESULTS AND DISCUSSION

5.1 Effect Of Shaking Speed On CF Production

Fig.5.1 shows that there was only a very low level of CF activity when shaking speed was 100 rpm or higher. The highest CF activity was detected in still culture filtrates although bacterial growth was the least compared with other treatments.

5.2 Effect Of Oxygen On CF Production

To distinguish whether the inhibitory effect of shaking was due to the effect of mechanical impact or oxygen toxicity, two experiments were designed. The first experiment was to grow bacteria in still liquid culture but supplied with membrane sterilised oxygen through the bottom of the culture flask. The flow of oxygen gas was such that the impact of mechanical force on bacteria could be neglected. Although the bacteria grew very well under these conditions, no CF activity was detected in the culture filtrate. This clearly indicates that the inhibitory effect of shaking is due to increased oxygen supply rather than to mechanical impact. The results of a second experiment are shown in Fig.5.2. Different volumes of culture medium were added to McArtney bottles and inoculated to give the same bacterial density in each. The screw caps were tightly closed, so the oxygen available to the bacteria in the bottles is in inverse proportion to the volume of the medium. Maximal CF activity was detected in the bottle that contained 10 ml of medium. Both oversupply and undersupply of oxygen repressed CF biosynthesis. This cannot be explained by an ~~a~~ffect on growth e (Fig.5 2). Clearly, oxygen is needed not only for the growth of bacteria but also for the synthesis of CF but too much oxygen represses its production.

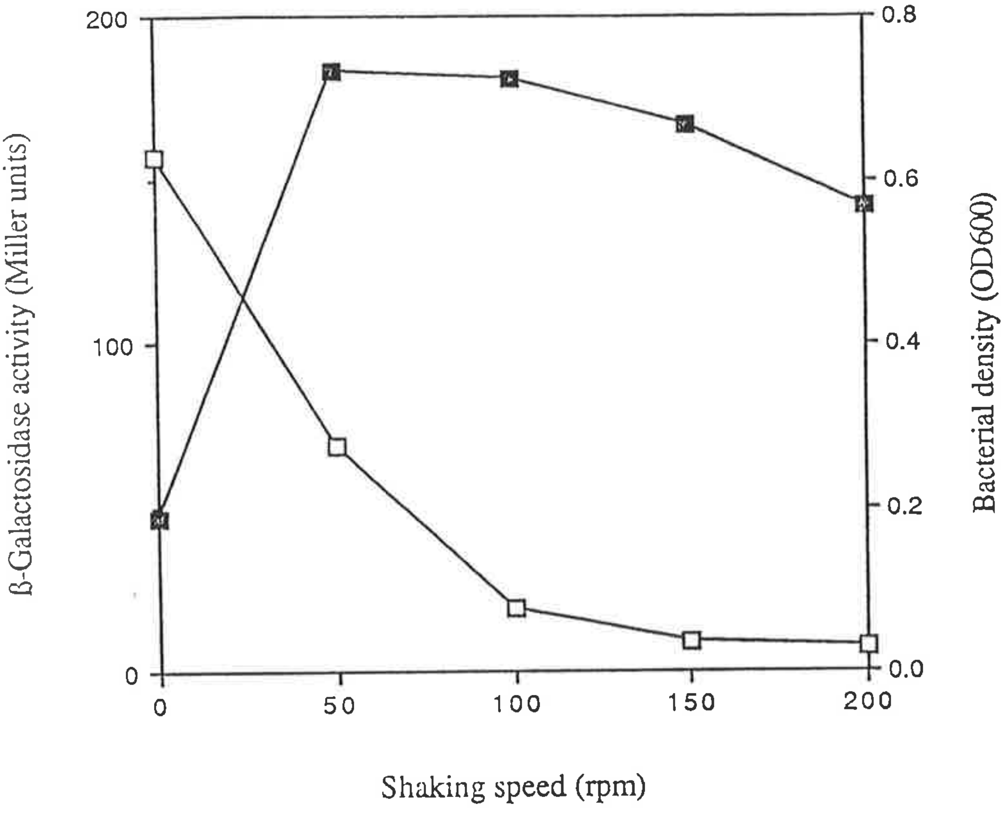


Fig. 5.1 Effect of shaking on CF production by *A. tumefaciens* octopine strain A6 in liquid cultures. Symbols: bacterial density (■), ß-galactosidase activity (❑).

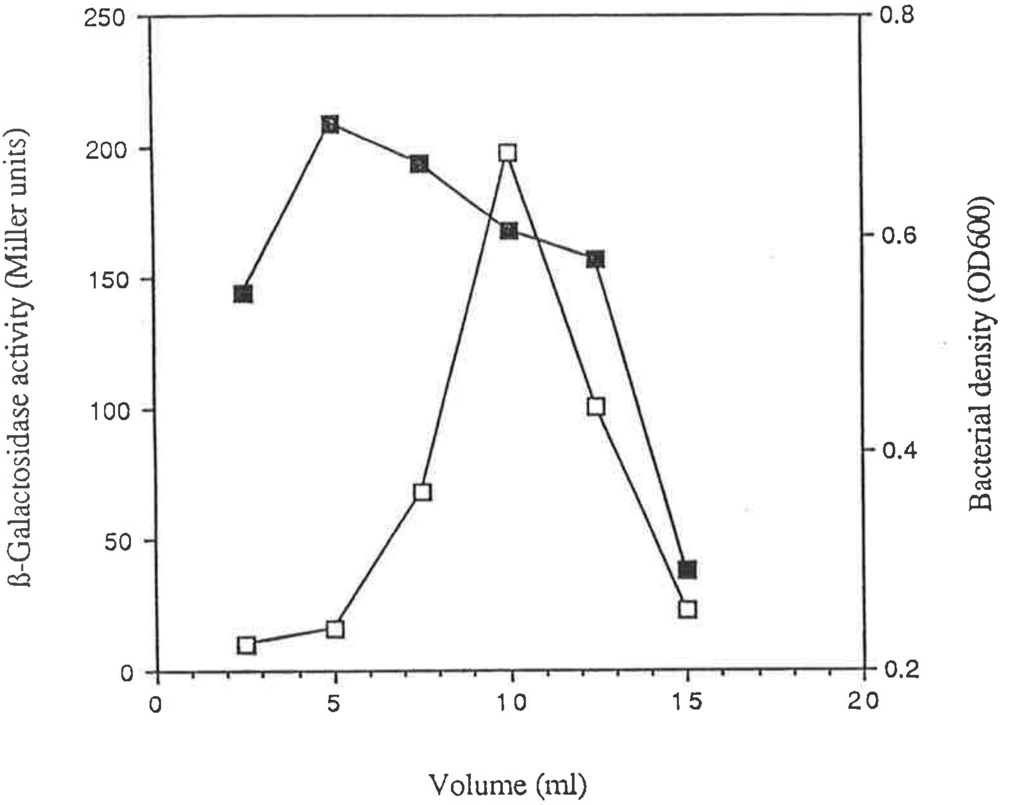


Fig.5.2 Effect of oxygen on CF biosynthesis and growth of *A. tumefaciens* octopine strain A6. Symbols: bacterial density (■); ß-galactosidase activity (□).

TABLE 5.1 Effect of $OH^{\cdot}$ scavengers on CF biosynthesis in shaking liquid cultures

Treatments	ß-Galactosidase activity (Miller units)	Bacterial density (OD_{600})
Control*	15.91	0.623
+ Ethanol (1%)	83.54	0.587
+ Butanol (1%)	66.53	0.571
+ Glucose (1%)	246.64	0.736
+ Mannitol (1%)	253.89	0.758
+ Tris (11 mM)	208.44	0.664
+ Catalase (1 mg/ml)	186.55	0.685
+ Denatured catalase (1 mg/ml)	14.74	0.636
+ EDTA (200 µM)	309.96	0.464

* Control: octopine induction medium.

5.3 Effect Of Free Radical Scavengers On CF Production

The biochemical mechanisms responsible for oxygen toxicity include lipid peroxidation and the generation of H_2O_2 plus the superoxide radical, O_2^-. In biochemical systems, O_2^- and H_2O_2 react together to form the hydroxyl radical, $OH^{\cdot}$ which is by far the most reactive oxidising agent known (Halliwell and Gutteridge, 1986; Reviewed by Sutherland, 1991). Table 5.1 shows that some powerful hydroxyl radical scavengers such as mannitol, glucose, and Tris (Halliwell and Gutteridge, 1986; Reviewed by Sutherland, 1991) strongly counteracted the inhibitory effects. Ethanol and Butanol which are weak free radical scavengers (Halliwell and Gutteridge, 1986;

Reviewed by Sutherland, 1991) had less effect. Inhibition of CF biosynthesis in the liquid shake culture was also reversed by catalase but not by heat-denatured catalase, which suggests that H_2O_2 was the source of the $OH^{\cdot}$ radicals responsible for the inhibition.

5.4 Effect Of Iron On CF Production

The iron chelator EDTA can prevent generation of hydroxyl radicals from iron salts (Gutteridge, 1984; Gutteridge et al, 1979) and Table 5.1 shows that it has a dramatic effect on the reversal of the inhibition. In our standard medium, the concentration of Fe_2SO_4 is 17 μM. Data in Fig.5.3 further confirm the involvement of iron in the inhibitory effect of oxygen on CF production. The higher the concentration of iron in the liquid medium, the less CF activity can be detected, even though bacterial growth is affected when the concentration of iron is too low.

5.5 Initiation Of CF Production After Shaking_ Is Stopped

Hydroxyl radicals have only a very brief half-life (Reviewed by Erich, 1982; by Sutherland, 1991). It is therefore predictable that CF production by agrobacteria should begin if shaking is stopped and is followed by still culture. Fig.5.4 shows that this is the case.

Several primary targets of oxygen radicals have been observed in cells, including membrane lipids and proteins, DNA, glyceraldehyde-3-PO_4 dehydrogenase (GAPDH) of the glycolytic pathway, mitochondria, and cytoskeletal elements (Cochrane et al, 1988). However, oxygen radicals seem more likely to regulate the induction of CF biosynthesis by opines in *A. tumefaciens* rather than to modify the chemical structure of CF since the conjugal transfer constitutive (Tra^{c}) strains of *A. tumefaciens* can produce CF constitutively in shaking liquid cultures (Zhang and Kerr, 1991; Chapter 3).

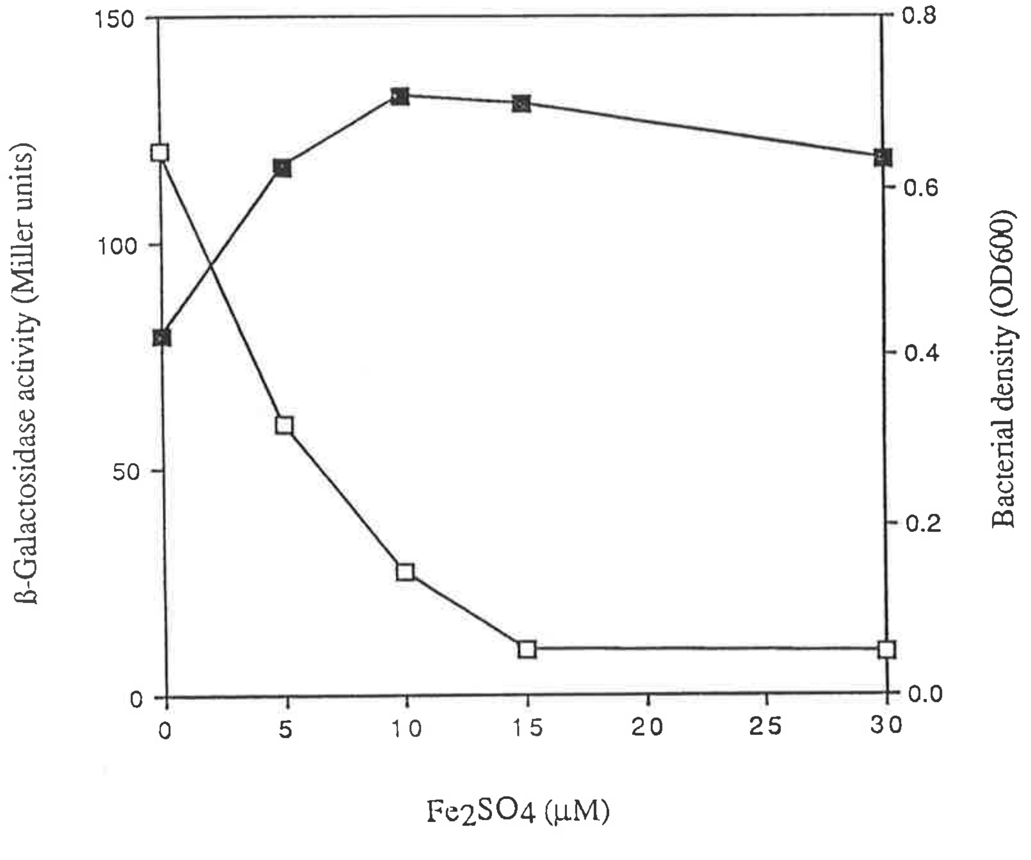


Fig.5.3 Effect of iron concentrations on *A. tumefaciens* octopine strain A6 growth and CF production in shaking liquid cultures. Symbols: bacterial density (■), ß-galactosidase activity (❑).

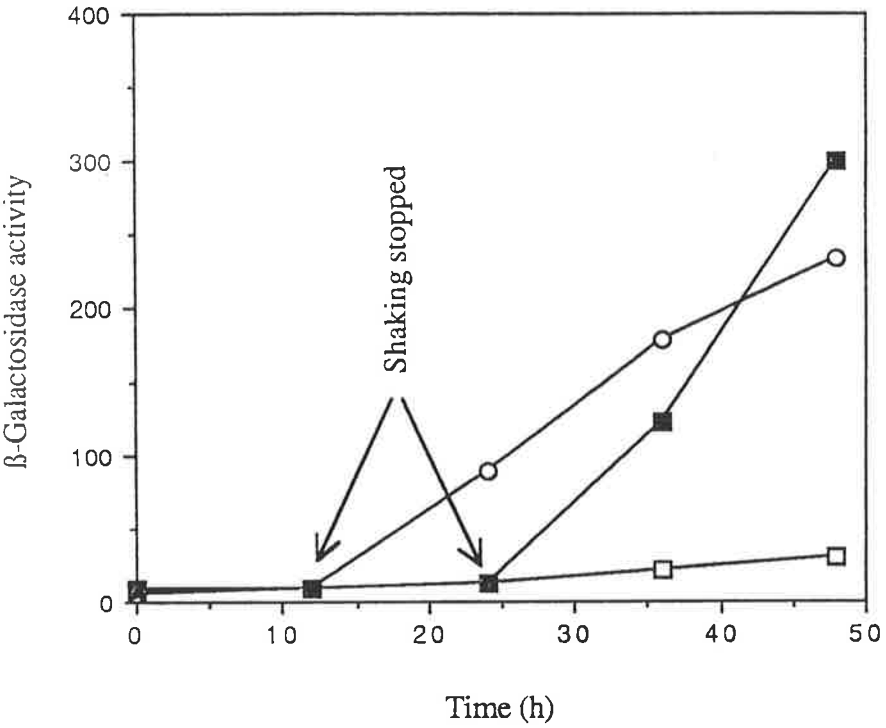


Fig.5.4 Start of CF production by strain A6 in liquid cultures after shaking was stopped. Symbols: shaking liquid culture (□); shaking was stoped after 12 h growth (○); shaking was stopped after 24 h growth (■).

CF was identified as *N*-(ß-oxo-octan-1-oyl)-L-homoserine lactone (Zhang et al, 1993; Chapter 4) and is structurally very similar to the autoinducer of bioluminescence in the markedly dissimilar bacterium, *Vibrio fischeri* (Eberhard et al, 1981). It is no surprise that similar factors including oxygen availability (Ruby et al, 1976) and iron content (Haygood and Nealson, 1984) were also found to be involved in the regulation of autoinduction of bioluminescence in *V. fischeri*, but the involvement of oxygen radicals was not established.

The biological significance of CF regulation by free radical oxygen is still unclear. One interesting possibility is that free radical oxygen is acting as a signal in the interaction between *Agrobacterium* and plant cells. There are two DNA transfer processes associated with the Ti plasmid of *A. tumefaciens*, viz. T-DNA transfer (Reviewed by Zambryski, 1988) and Ti plasmid conjugal transfer (Reviewed by Kerr and Ellis, 1982); for effective T-DNA transfer to the plant to occur, it would seem reasonable that Ti plasmid conjugal transfer is first inactivated. As T-DNA transfer requires fresh wounding (Reviewed by Zambryski, 1988) and as wounding results in the production of oxygen radicals as a stress response (Salin and Bridges, 1981; Reviewed by Erich, 1982; and by Sutherland, 1991), it is feasible that these free radicals may then inactivate bacterial conjugation.

CONCLUSION

Conjugation factor production by *Agrobacterium tumefaciens* was inhibited in shaking liquid cultures. Inhibition was reversed either by limiting oxygen supply, adding catalase (but not denatured catalase) or adding EDTA to the cultures. Hydroxyl radical scavengers also showed significant counter-inhibition. Results indicate that oxygen radicals are involved in the regulation of conjugation factor biosynthsis.

CHAPTER.6. RAPID PURIFICATIION OF TI PLASMIDS FROM *AGROGACTERIUM* BY ETHIDIUM BROMIDE TREATMENT AND PHENOL EXTRACTION

INTRODUCTION

It was shown in Chapter 3 that the Ti plasmid of *Agrobacterium tumefaciens* is required for biosynthesis of conjugation factor. To facilitate molecular studies on the biological functions encoded by the Ti plasmid, an efficient method is needed to purify plasmid DNA from *Agrobacterium.* Various rapid procedures to prepare relatively pure plasmid DNA from small volumes of *E. coli* cultures have been developed for restriction mapping purposes (He, *et al.* 1989, Holmes & Quigley, 1981, and Saunders & Burke, 1990) but for purification of Ti plasmids from *Agrobacterium*, these procedures often result in low yield or an impure product. This is possibly due to the fact that the Ti plasmid has a low copy number and is large which makes it more fragile.

The standard method for preparative purification of plasmid DNA is isopycnic ultracentrifugation through CsCl-ethidium bromide gradients. The principle of this method is based on the fact that ethidium bromide (EtBr) can change the density of DNA by intercalating between adjacent base pairs and much more EtBr intercalates in linear DNA than in supercoiled DNA (reviewed by Pecq, 1971). This is the only reliable method presently available to purify Ti plasmids from *Agrobacterium* but it is time consuming and requires expensive instruments and chemicals. In fact, it was the breakdown of the only ultracentrifuge in the laboratory that initiated exploration of the possibility to develop a simple method.

It was noted that EtBr is much more soluble in phenol than in H_2O, in contrast to DNA. It seemed possible that linear DNA with high EtBr intercalation might be soluble in phenol whereas supercoiled plasmid DNA with low EtBr intercalation would remain water soluble. This proved to be so. This Chapter describes a rapid small scale EtBr-phenol extraction procedure which exploits the differential solubility in phenol of plasmid DNA compared with linear DNA and RNA after EtBr treatment. The method is simple, rapid and efficient and can be scaled up for the preparative isolation of plasmids from litre cultures with quantitative yields.

MATERIALS AND METHODS

Reagents And Bacterial Strains

All chemicals were obtained from SIGMA or BDH and were at least analytical grade. Solution I (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0), solution II (0.2 N NaOH, 1% SDS), solution III (3M potassium acetate, adjusted to pH4.8 with glacial acetic acid) and phenol solution (phenol:chloroform:isoamylalcohol, 25:24:1, containing 0.1% 8-Hydroxyquinoline, pH<7.6) were prepared as described by Maniatis et al.(1982). *A.tumefaciens* strain A6 and *Escherichia coli* strains HB101(pVK102) and HB101(pBIN19) were used in this study.

Small Scale Plasmid Isolation And Purification Procedure

The small scale isolation procedure is based on the modified alkaline procedure of Currier and Nester (1976). Bacteria were grown for 24 h in 10 ml of YEB medium (Van Larebeke et al., 1977) at 28° C. They were collected by centrifugation (11,000g) in 10 ml tubes for 2 min and transferred to a 2 ml tube after resuspention in 1 ml LTE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), 100 µl 5 M NaCl and 20 µl 10% Na Sarkosyl. The bacterial suspension was mixed gently and centrifuged (11,000 g) for 2 min. The pellet was resuspended in 200 µl solution I containing 2 mg/ml lysozyme and placed on ice for 5 min. Bacteria were lysed by adding 400 µl solution II , inverting gently to mix and leaving at 37° C for 10 min. The solution was neutralised by adding

100 µl 2 M Tris (pH7.0), mixed well but gently by inversion and then let stand for 30 min at room temperature; 100 µl 5 M NaCl and 800 µl phenol solution were added and mixed gently by inversion for 5 min. The aqueous phase was collected and transferred to a fresh 2 ml tube after centrifugation for 10 min. The crude DNA was precipitated by adding an equal volume of isopropanol, mixing and centrifuging for 15 min. The pellet was washed once with 1 ml 70% ethanol and dried in air for 2 min.

For purification, the following EtBr-phenol extraction procedure was used. The pellet was dissolved by adding 100 µl solution I, 170 µl sterile H_2O and 10 µl 2 M Tris, mixing gently and leaving at 50° C for 5 min . After adding a mixture of 20 µl 2 N NaOH and 150 µl ice-cold solution III, the solution was left on ice for 5 min. Then 40 µl 1% EtBr was added, mixed gently and, left on ice for 30 min; after gentle mixing, the solution was centrifuged for 5 min. The supernatant was extracted using an equal volume of phenol solution by slowly inverting for 5 min. The aqueous phase was collected by centrifugation for 5 min and reextracted for 3 min with the same volume of chloroform. Plasmid DNA was precipitated by adding an equal volume of isopropanol, mixing and centrifuging for 15 min. The pellet was washed twice with 70% ethanol and dried in vacuo. It was then dissolved in 20 µl LTE. The amount of Ti plasmid DNA in solution is suitable for three restriction enzyme digests.

Preparative Purification Of Plasmid DNA

The large scale alkali lysis method (Maniatis et al, 1982) and the modified alkali lysis method (Currier & Nester 1976) were followed to isolate plasmid DNA from 1 litre cultures of *E. coli* and *A. tumefaciens* respectively. The plasmid pellet precipitated by ethanol was dissolved in 4 ml of solution I, 6.8 ml of sterile H_2O and 400 ml of 2 M Tris buffer. The dissolved DNA solution was distributed equally into four 10 ml disposable plastic centrifuge tubes. To each tube, 200 µl of 2 N NaOH solution was added and left at room temperature for 5 min before neutralisation with 1.5 ml of solution

III. Then the EtBr-phenol extraction procedures described above were followed, using ten times the amount of EtBr.

Effect Of Ethydium Bromide On The Partition Of Linear And Plasmid DNA Into Phenol And Aqueous Phases

The EtBr-phenol extraction procedure described above was followed to study the effect of EtBr on the partition of plasmid DNA and linear DNA into phenol and aqueous phases. Linear salmon sperm DNA was labelled with ^{32}P-dCTP using a nick translation kit (BRESA) according to the manufacturer's instructions. The radioactivity of ^{32}P-labelled linear DNA was about 1.2×10^7 cpm/µg. After EtBr-phenol extraction, radioactivity in phenol and aqueous phases was measured in a Beckman LS 5000 TD Liquid Scintillation System.

RESULTS AND DISCUSSION

6.1 Effect Of EtBr On The Partition Of Plasmid DNA Into Aqueous And Phenol Phases

Different sized plasmids purified from *A. tumefaciens* and *E. coli* were used for the assessment of recovery of plasmid DNA in aqueous phase after EtBr-phenol extraction. Table 6.1 shows that all undenatured plasmid DNA was recovered with an efficiency of more than 90%. In fact, recovery of small sized plasmid DNA was almost 100%. However, following denaturation, in which the supercoiled structure of plasmid DNA was destroyed by heat, about 90% of all plasmid DNA was removed by EtBr-phenol extraction.

TABLE 6.1 Recovery of plasmid DNA after EtBr-phenol extraction

Plasmid and size	Percentage recovery *	
	Supercoiled plasmid	Denatured plasmid†
pBIN19 (10 kb)	100	9.8
pVK102 (23 kb)	99.2	9.7
pTiA6 (190 kb)	91.6	9.4

* The experiments were repeated three times, using 24 μg of plasmid DNA for each experiment.

† Plasmid DNA was denatured by heating in boiling water for 10 min then placing in ice water for 5 min.

6.2 Effect Of EtBr On The Partition Of Linear DNA Into Aqueous And Phenol Phases

To determine the effect of EtBr on the partition of linear DNA into aqueous and phenol phases, ^{32}P-labelled salmon sperm DNA was used. Fig.6.1 shows that EtBr has a marked effect on the partition of linear DNA into the phenol phase. Nearly all linear DNA remained in the aqueous phase if not treated with EtBr before phenol extraction, whereas following 30 min EtBr treatment, 87.4% of linear DNA was present in the

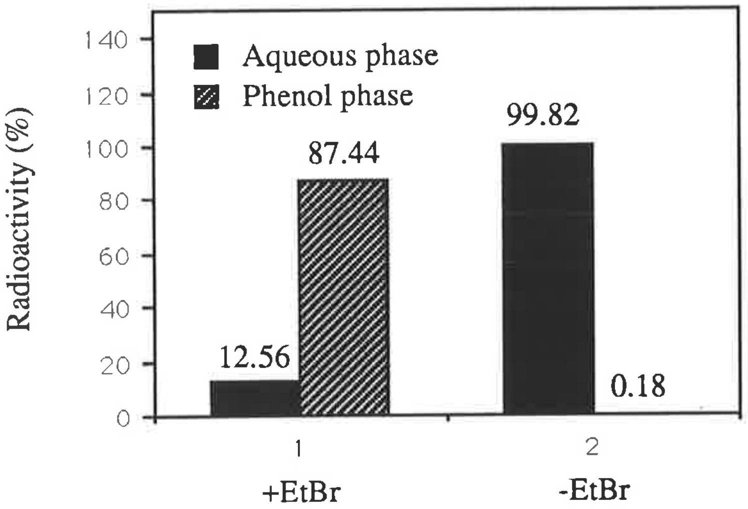
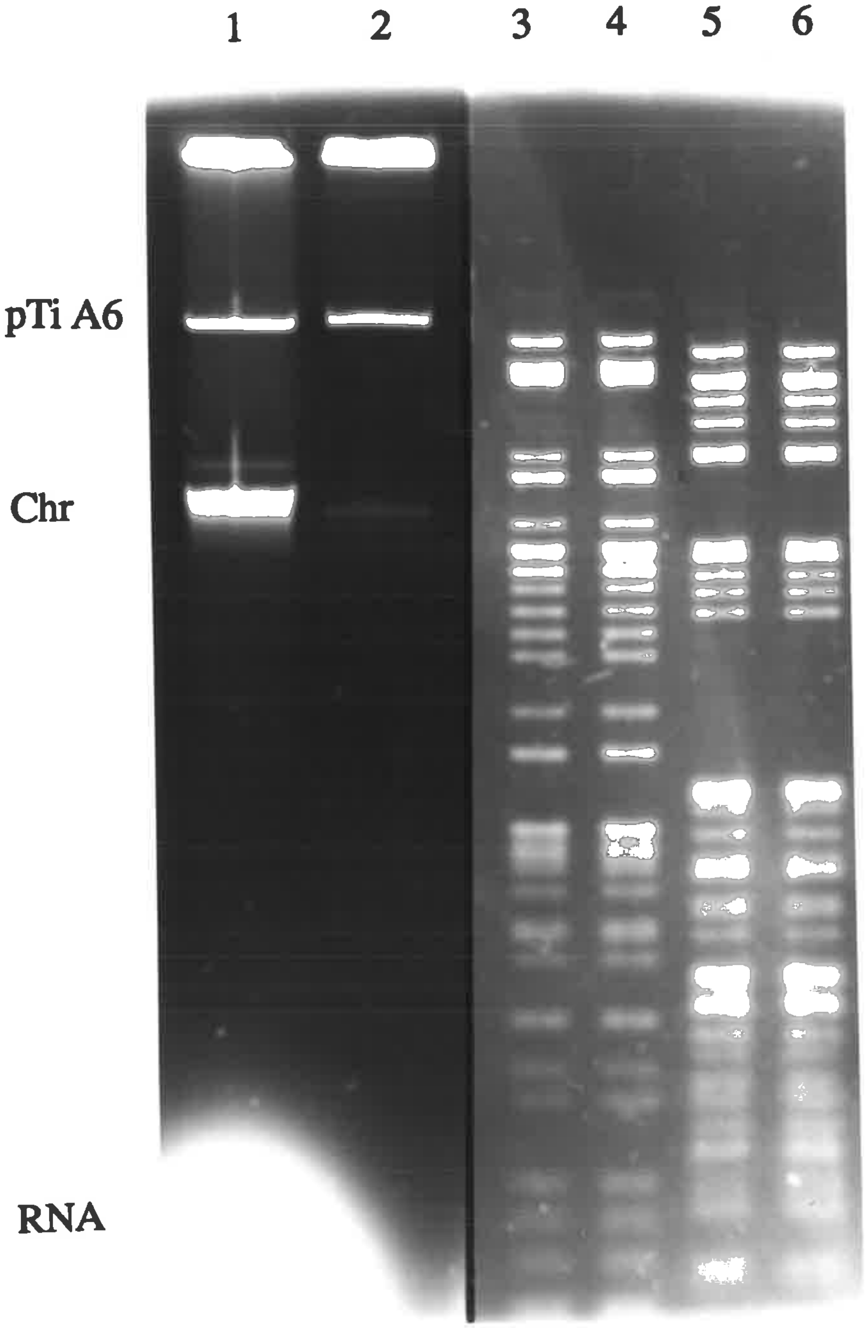


Fig. 6. 1 Effect of ethydium bromide on the partition of linear DNA into phenol and aqueous phases. The experiment was repeated three times using a mixture of 5 ml of ^{32}P-labelled linear DNA (about 20 ng) and 80 mg unlabelled linear DNA.

Fig.6.2. Ti Plasmid profiles and restriction enzyme digestion patterns. Lanes 1 and 2 show pTi A6 from *Agrobacterium* before and after EtBr-phenol extraction. Lanes 3 and 5 are *Hin*dIII and *Eco*R1 digestion patterns respectively of pTi A6 purified by CsCl-EtBr gradient ultracentrifugation; Lanes 4 and 6 are the same enzyme digestion patterns of pTi A6 purified by EtBr-phenol extraction; 6 μg plasmid DNA was digested by 3 units restriction enzyme at 37° C for 30 min. Chr: chromosomal DNA.


1
2
3
4
5
6
pTi A6
Chr
RNA

phenol phase. Fractionation of the phenol phase indicated that the majority of the ^{32}P labelled linear DNA was in the top layer of the phenol phase.

6.3 Yield And Quality Of Purified Plasmid DNA

Fig.6.2 shows a comparision of the DNA isolated before and after EtBr-phenol extraction and the restriction enzyme digestion patterns of the purified plasmid DNA; only a trace of genomic DNA and RNA can be detected after EtBr-phenol extraction. Fig.6.2 also shows that purified plasmid DNA is subject to complete digestion by *Hin*dIII and *Eco*R1.

The efficiency of Ti plasmid extraction by the EtBr-phenol protocol and by CsCl-EtBr gradient ultracentrifugation was compared. The yields of Ti plasmid were about 180 μg to 300 μg per litre varying from batch to batch after ultracentrifugation. The yield of Ti plasmid by the new method was less variable and much higher, being around 2500 μg/l each run. This procedure has been successfully used in this laboratory for more than one year to purify Ti plasmids ranging from 170 to 200 kb for genetic mapping and DNA cloning, with quality comparable to and yield 8 times better than is obtained by EtBr-CsCl density gradient ultracentrifugation.

CONCLUSION

An efficient method is described for the purification of Ti plasmid DNA from *Agrobacterium*. The procedure is based on the relative binding capacity of ethidium bromide to supercoiled plasmid DNA and linear DNA and on the high solubility of ethidium bromide in phenol. Following treatment with ethidium bromide more than 87% of linear chromosomal DNA and most of the RNA were present in the phenol phase, while 91% of Ti plasmid DNA was recovered from the aqueous phase. The Ti plasmid DNA was pure enough for restriction endonuclease analysis and cloning. The procedure is simple, fast and provides eight times higher yield over the standard isopycnic ultracentrifugation method.

CHAPTER 7. MOLECULAR CLONING OF THE TI PLASMID ENCODED *cfsA* GENE AND CF OVERPRODUCTION BY INCREASING COPY NUMBER OF *cfsA* GENE

INTRODUCTION

A previously unknown conjugation factor (CF) has been identified as *N*-ß-oxo-octanoyl-L-homoserine lactone (Zhang et al., 1993; Chapter 4). This is, to our knowledge, the first example of a second messenger signal molecule discovered in a bacterial conjugation system. The biosynthesis of CF was found to be regulated by both octopine and oxygen radicals (Zhang and Kerr, 1991; Chapters 3 and 5).

CF is structurally very similar to the *lux* autoinducer in *Vibrio fischeri* (Eberhard et al., 1981), indicating that *N*-acyl-homoserine lactones may be a conserved group of signal molecules in gene regulation. In fact, a very recent report indicates that the *lux* autoinducer also regulates antibiotic biosynthesis in *Erwinia* (Bainton et al., 1992). Another *N*-acyl-homoserine lactone, *N*-ß-hydroxy-butanoyl-L-homoserine lactone was found to be the autoinducer of bioluminescence in *V. harveyi* (Cao and Meighen, 1989).

This Chapter describes the molecular cloning and characterisation of the principle genes for the biosynthesis of the conjugation factor encoded by the Ti plasmid in octopine strains of *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial Strains And Plasmids

Agrobacterium strains A6 and K749 have been described in Chapter 2. *Agrobacterium* strain K588 containing pTiB6Tra^c is a conjugal transfer and CF production constitutive mutant. Ti plasmid minus *Agrobacterium* strains NT1 and Ach5C1 obtained from S. Farrand are Ti plasmid cured derivatives of nopaline strain C58 (Watson et al., 1975) and octopine strain Ach5 (Klapwijk et al., 1979) respectively. Strain M106 has been described in Chapter 8 (Tables 8.1 and 8.2). Plasmid pAL4404 supplied by J. Ellis is a pTiAch5 deletion mutant (Hoekema et al., 1983) and is illustrated in Fig.7.3. Plasmid pJS400::*Kpn*I-1 which was constructed and supplied by S. Farrand is a plasmid clone in which the *Kpn*I fragment No.1 of pTi15955 carrying the Inc/Ori region was cloned in the plasmid vector pJS400 (Dessaux et al., 1987). Plasmid pLZK1 was constructed in this study in which the *Kpn*I fragment No.1 of pTiB6Tra^c covering the Inc/Ori region was cloned into the *Kpn*I site of a broad-host-range vector pDSK519 (Keen et al., 1988). Vector pDSK519 originated from the IncQ plasmid pRSF1010 (Keen et al. 1988) and therefore it is compatible with the IncP-1 vector pVK102 (Knauf and Nester, 1982). *Escherichia coli* strain HB101 (Boyer and Roulland-Dussoix, 1969) was used in the cosmid library construction and matings. pRK2013 (Ditta et al., 1980) was used as a helper plasmid in triparental matings. Other plasmids used in the experiments described in this Chapter are described in Fig.7.4.

Media And Growth Conditions

Minimal medium with 1 mg of $(NH_4)_2SO_4$ per ml as nitrogen source and YEA medium have been described (Chapter 2). When indicated, an octopine concentration of 200 µg per ml was used to replace $(NH_4)_2SO_4$ as a sole nitrogen source. BM medium (per litre containing mannitol, 10 g; $(NH_4)_2SO_4$, 0.1 g; $Na_2HPO_4 \cdot 12H_2O$, 0.45 g; $CaCl_2$, 0.04 g; $FeCl_3$, 0.02 g; agar, 15 g; pH7.0) was used in triparental matings. Bromothymolblue indicator medium has been described (Hooykaas et al., 1979). *Agrobacterium* strains were grown at 28° C. *E. coli* strains were grown at 37° C in LB medium (Miller, 1972).

Chemicals

Synthesis of conjugation factor (*N*-ß-oxo-octanoyl-L-homoserine lactone) has been described in Chapter 4. X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) was from SIGMA; a stock solution of it was prepared (20 mg/ml in dimethylformamide) and stored at -20° C.

Construction Of A pTiB6Tra^c Cosmid Library

Plasmid DNA was purified as described in Chapter 6. The conjugal transfer constitutive mutant K588 (pTiB6Tra^c) was used in this experiment. A dephosphorylated partial *Hin*dIII digest of pTiB6Tra^c was cloned into the *Hin*dIII site of a broad host range cloning vector pVK102 (22 kb in size). The ligation mixture was packaged by using Gigapack^{TM} II XL Packaging Extract (STRATAGENE COMPANY) which preferentially packages those cosmid clones containing 25-29 kb inserts with high efficiency. A total of 180 cosmid clones were selected in the cosmid bank of pTiB6Tra^c.

DNA Probing

Probe DNA was labeled with ^{32}P-dCTP using a nick translation kit (BRESA) and colony hybridization was carried out as described by Maas (1983).

Construction Of Merodiploid Strains

Plasmid clones were mated into *Agrobacterium* strains by triparental matings as described by Maniatias et al. (1982) with selection on minimal BM medium supplemented with appropriate antibiotics. Merodiploidy was confirmed by plasmid DNA analysis.

Deletion Analysis Of pTiB6Tra^c cfsA Genes

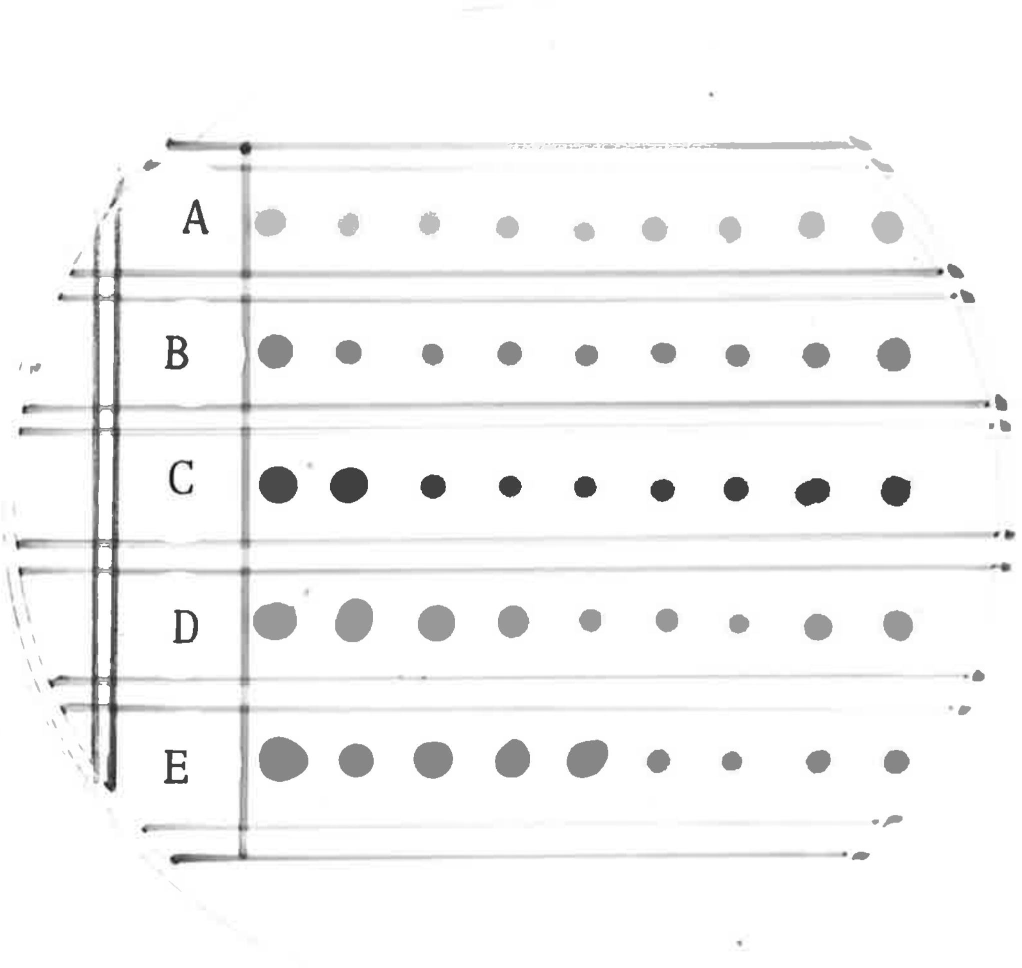
The *cfsA* clone pBK129 (in the vector pVK102) was partially digested with *Hin*dIII; the ends of the plasmid were religated and transformed into *E. coli* HB101. Individual colonies were picked and their plasmids analysed. Different sized deletion plasmids were selected and transferred into *Agrobacterium* strains by triparental mating and assayed for CF production.

Quantitative Determination Of Conjugation Factor

In Chapter 4 and Chapter 5, an enzymatic method was used to quantify CF activity in solutions and in bacterial culture filtrates. To assay CF production in living bacteria, this method is time consuming. For example a culture filtrate of CF producing strains has to be prepared before assaying for CF activity (Chapter5). As well, this assay becomes saturated at higher levels of CF, viz, when the concentration of CF rises to a certain point in the solution the reporter gene response plateaus (refer to Fig.4.10, Chapter 4).

In this Chapter, an exponential equation was fitted and used for quantitative determination of CF. Conjugation factor is diffusible in agar medium (Zhang and Kerr, 1991; Chapter 3), and this diffusive behaviour of CF can be described by an exponential mathematical equation. To determine this equation, a series of different concentration of synthetic conjugation factor was prepared, and 5 μl of each concentration was added to one end of an agar slice (1 cm in width) in a X-gal minimum medium plate (25 ml of melted agar minimum medium was poured into a plastic plate of 8 cm in diameter; 50 μl of X-gal in a concentration of 20mg/ml were spread on the surface of the medium after the plates had solidified). Fresh cultures (0.6 μl of $OD_{600} \approx 0.4$) of CF bioassay strain NT1(*traR; tra::lacZ749*) described in Chapter 4 was spotted at progressively further distances from the CF sample (illustrated in Fig. 7.1). The experiment was repeated 3 times. The plates were incubated at 28° C. A few hours later, blue colonies appeared,

Fig.7.1 A diffusion plate for quantitative determination of conjugation factor (*N*-ß-oxo-octanoyl-L-homoserine lactone). Different concentrations of conjugation factor (5 µl) were added to the squares on the left side of the plate, and a fresh culture of the conjugation factor bioassay strain NT1 (*traR, tra::lacZ*749) was spotted at regular intervals, onto the medium to the right side of the squares as described in text. Results were recorded after 24 h. Double lines indicate that a slice of agar has been removed. Concentration of CF used: A, negative control; B, 1 µM; C, 10 µM; D, 100 µM; E, 1000µM.


A
B
C
D
E

first in the area adjacent to CF and then gradually further away. After 24 h, the distance of the last induced (blue) colony from the origin in each agar slice was measured. The distance was defined as the diffusion distances of conjugation factor, because in this bioassay strain, the ß-galactosidase encoded by *lacZ* gene which was fused to the *tra* gene is expressed only when induced by CF (Piper et al., 1993; Zhang et al., 1993; Chapter 4). The amount of CF added and the corresponding diffusion distances in minimum agar medium are shown in Fig. 7.2. An exponential regression equation was then fitted using the diffusion distance (cm) as X_i and the amount (ng) of CF as Y_i. (Fig. 7.2):

$$Y = 0.67312 \times 10^{1.0357X} \qquad (7.1)$$

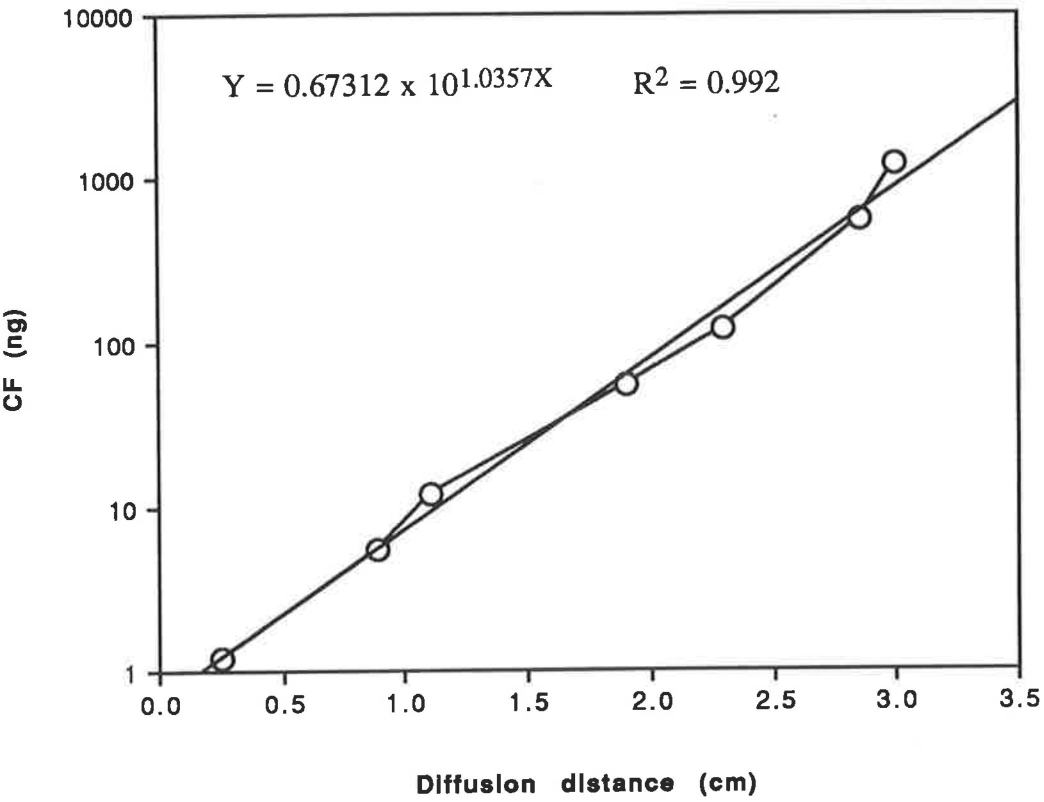
The coefficient of determination (R^2) of the above exponential equation is equal to 0.992 (Fig.7.2), indicating a very high correlation and making it suitable for the description of diffusion of conjugation factor in agar.

In the experiments described in this Chapter, the amount of conjugation factor produced by *Agrobacterium* strains was determined using the same experimental procedures except that instead of measuring the diffusion of a known concentration of CF, a small amount (2 µl of $OD_{600} \approx 0.8$) of fresh bacterial culture was spotted onto one end of the agar slice, and after 24 h incubation at 28° C, the distance (X) that the CF has diffused was measured. The amount of CF (ng) produced by the bacteria was calculated using equation (7.1).

Octopine Catabolism

The bromothymolblue indicator medium was used to distinguish octopine-utilising agrobacteria from non-utilisers as described by Hooykaas et al. (1979).

Fig.7.2 An exponential curve describing diffusion of conjugation factor in agar. Y = the amount (ng) of CF; X = the diffusion distance (cm) of CF (represented by the distance of blue colonies from the origin).


Y = 0.67312 x 10^1.0357X
R^2 = 0.992
10000
1000
100
10
1
CF (ng)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Diffusion distance (cm)

RESULTS AND DISCUSSION

7.1 Strategic Approach

The following approach has been used to isolate *cfs* genes from *A. tumefaciens*. Here *cfs* genes are defined as all those genes involved in CF biosynthesis.

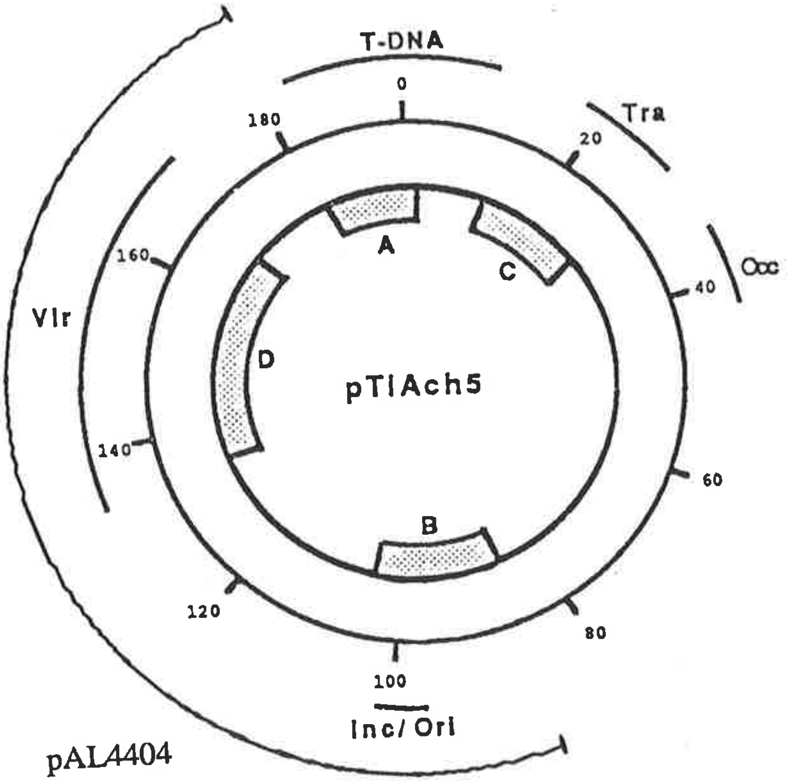
The approach was to transform a plasmidless and CF$^-$ *Agrobacterium* strain with "candidate" cosmid clones from the Ti plasmid and then assay for CF production. The fact that the Ti plasmid is needed for CF production in *A. tumefaciens* and that both octopine and nopaline strains can produce the same or similar CF (Chapter 3; Zhang and Kerr, 1991) indicate that at least some CF genes are located on the common regions of octopine and nopaline Ti plasmids. It has been reported that there are four homologous regions between an octopine (pTiAch5) and a nopaline (pTiC58) Ti plasmid and these common regions represent approximately 30% of the Ti plasmid genome (Engler et al., 1981; Fig.7.3). Therefore, the plasmid clones that cover these common regions were selected and used as "candidates" to test for CF production.

7.2 A cfs Locus In The Occ/Tra Region Of pTiB6Tra^c Enables Wild Type Strain A6 To Produce Conjugation Factor Constitutively

CF production is induced by octopine (Chapter 3). The fact that a Tn*5* insertional mutation in the Occ/Tra region of strain M106 causes loss of Occ, Tra and Cfs functions (refer to Chapter 8, Tables 8.1 and 8.2), indicates that there is probably a common regulatory gene for these three functions. It seems rational to assume that some CF structural genes are located in the Occ/Tra region (refer to Fig.7.3) because structural genes are often found in the same region as the regulatory gene(s).

The cosmid bank of pTiB6Tra^c was screened for clones covering the Occ/Tra region using the fragments adjacent to the above Tn*5* insertion in strain M106 as a probe. Three clones (pBK207, pBK129, pBK218) that were homologues to the probe were

Fig.7.3 Map of octopine-type Ti plasmid pTiAch5 showing regions (shadowed boxes A, B, C, and D) of homology with nopaline-type Ti plasmid pTiC58. The corresponding regions contained in plasmid pAL4404, a deletion derivative of pTiAch5, are also indicated. The Ti plasmid map (kb coordinates), their functional regions, and homologies are according to Clare et al. (1990). The map of pAL4404 is based on Ooms et al. (1982), and Hoekema et al. (1983). Abbreviations used: Tra, conjugal transfer; Occ, octopine catabolism; Inc/Ori, incompatibility/origin of replication; Vir, virulence region; T-DNA, transfer DNA.


T-DNA
0
180
20
Tra
Occ
40
A
C
160
Vir
D
pTiAch5
140
60
B
120
80
100
Inc/Ori
pAL4404

mated into different *Agrobacterium* strains by triparental mating. The transconjugants were then tested for CF production and octopine catabolism.

The results in Table 7.1 show that all three cosmid clones encode octopine catabolism function. But none of them can confer on strain K749 the ability to produce conjugation factor. The possibility that CF production is encoded by more than one genetic locus on the Ti plasmid was then considered. To test this, the above three cosmid clones were transferred individually into strain A6 which contains a wild type Ti plasmid pTi A6; then CF production was tested with or without the presence of octopine. Strain A6 (pTi A6) can produce CF only when induced by octopine, but strains A6 (pTiA6; pBK129) and A6 (pTiA6; pBK218) can produce conjugation factor constitutively (Table 7.1). These data suggest that cosmid clones pBK129 and pBK218 contain a genetic locus which encodes constitutive expression of CF biosynthesis genes. But this locus is not the only one involved in CF biosynthesis, it needs to cooperate with other *cfs* locus(loci) which is likely located on other parts of the Ti plasmid.

7.3 Conjugation Factor Biosynthesis Is Encoded By Two Or More Loci In The Ti Plasmid

Results of the previous experiment indicated that more than one locus was involved in CF biosynthesis. To test this possibility, different clones from the Ti plasmid that cover the common regions of octopine and nopaline Ti plasmids were tested for CF production in combination with the cosmid clone pBK129. Data in Table 7.2 show that only strains LBA4404 (pAL4404; pBK129) and NT1 (pTiB6Tra^{c}) can produce conjugation factor. Strain LBA4404 has the same chromosomal background as Ach5C1, therefore, the second *cfs* locus needed for conjugation factor production must be carried by plasmid pAL4404. Plasmid pAL4404 is a deletion derivative of pTiAch5 (Hoekema et al., 1983) and is about 90 kb in size (Fig. 7.3). It contains only two common regions, viz. Vir and Inc/Ori regions, conserved on both octopine and nopaline Ti plasmids (Fig. 7.3). It seems likely that the second *cfs* locus is located within or near the Vir region, because two plasmid clones pJS400::*Kpn*I-1 and pLZK1 containing Inc/Ori regions from pTi15955 (Dessaux et al., 1987) and from pTiB6Tra^{c} respectively did not show any CF production in the background of NT1 (pBK129) (Table 7.2). This possibility is greatly strengthened by the evidence that transposon mutagenesis in the *vir* genes decreased significantly the conjugal transfer efficiency of the Ti plasmid (Gelvin and Habeck, 1990; Steck and Kado, 1990).

TABLE 7.1 The Occ/Tra region from pTiB6Tra^{c} enables wild type strain A6 to produce conjugation factor constitutively

Strains and plasmids	CF production (ng)		Octopine
	+ Octopine	- Octopine	catabolism
K749	0	0	-
K749(pTiB6Tra^{c})	21.3	18.7	+
K749(pBK107)	0	0	+
K749(pBK129)	0	0	+
K749(pBK218)	0	0	+
A6(pTiA6)	5.8	0	+
A6(pTi A6; pBK107)	5.9	0	+
A6(pTiA6; pBK129)	70.7	14.95	+
A6(pTiA6; pBK218)	68.2	14.05	+

TABLE 7.2 A second locus on Ti plasmid is needed for conjugation factor biosynthesis

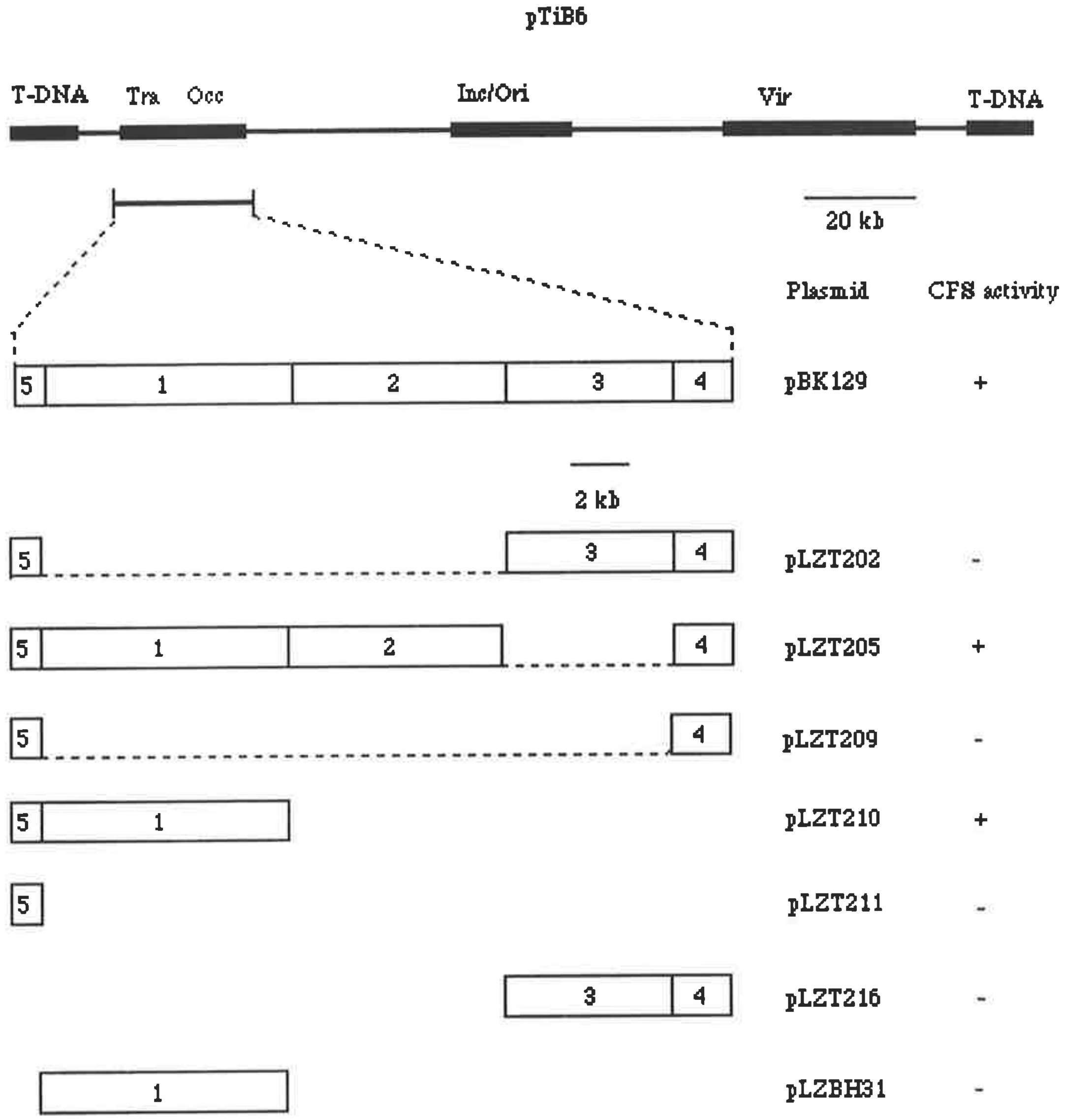
Strains and Plasmids*	CF production
Ach5C1(Cr)	0
Ach5C1(Cr; pBK129)	0
LBA4404(Cr; pAL4404; pBK129)	24.08
NT1	0
NT1(pTiB6Tra^{C})	21.67
NT1(pJS400::*Kpn*I-1)	0
NT1(pJS400::*Kpn*I-1; pBK129)	0
NT1(pLZK1; pBK129)	0

* Abbreviation: Cr, Cryptic plasmid.

7.4 Subcloning The cfsA Locus

A *Hin*dIII physical map of cosmid pBK129 was constructed by the deletion cloning (Fig. 7.4). Plasmid DNA of pBK129 was purified and partially digested with *Hin*dIII. Different sized deletion subclones were ligated and transformed into *E. coli* strain HB101 and then introduced into *Agrobacterium* strain LBA4404 (pAL4404) by triparental mating. Data in Fig.7.4 show that in combination with plasmid pAL4404, the smallest deletion clone which can produce conjugation factor is pLZT210 which is 10.2 kb in size and contains *Hin*dIII fragments No.1 and No.5 of the cosmid clone pBK129. The deletion clone pLZT211 and *Hin*dIII subclone pLZBH31, that contain only *Hin*dIII fragment No.5 or No.1 of cosmid clone pBK129 respectively, fail to produce CF in the

Fig.7.4 Deletion analysis of the *cfsA* locus. Map of pTiB6 is based on De Greve et al. (1981). The physical map (*Hin*dIII) of cosmid clone pBK129 was constructed by deletion analysis. *Hin*dIII fragments of pBK129 were numbered in order of size. Deletions of pBK129 were prepared, mated into *Agrobacterium* strain LBA4404 (pAL4404), and assayed for CF synthesis (CFS activity) as described in Materials and Methods.


pTiB6
T-DNA
Tra
Occ
Inc/Ori
Vir
T-DNA
20 kb
Plasmid
CFS activity
5
1
2
3
4
pBK129
+
2 kb
pLZT202
-
pLZT205
+
pLZT209
-
pLZT210
+
pLZT211
-
pLZT216
-
pLZBH31
-

background of LBA4404 (pAL4404). Therefore, the *cfsA* locus is located in the *Hin*dIII fragments No.1 and 5 of the cosmid clone pBK129.

7.5 CF Overproduction By Increasing Copy Number Of The cfsA Locus

Yield of gene product can usually be improved by increasing the copy number of the structural gene. The Ti plasmid is a single copy number plasmid in *Agrobacterium* (Gallie, et al., 1985). When cosmid clones pBK129 and pLZT210 containing the *cfsA* locus were transferred into strain K588(pTiB6Tra^c) by triparental mating, the yield of CF production was increased by 3.9 to 4.9 times (Table 7. 3). The cosmid vector pVK102 (Knauf and Nester, 1982) used in this experiment is a derivative of the bacterial plasmid RK2 which exists at approximately 5.3 copies per chromosomal equivalent in *E. coli* (Figurski et al., 1979). Assuming that the same copy number occurs in *Agrobacterium*, the incremental increases of CF production and the copy number of the *cfsA* locus are roughly proportional. It is therefore likely that the *cfsA* locus is a structural gene for the conjugation factor.

Strain LBA4404 (pAL4404; pBK129) which probably contains 5 copies of the *cfsA* locus produced only 24.08 ng (per colony/24 h) conjugation factor (Table 7.2), and is about the same level as that produced by strain K749(pTiB6Tra^c) which has only 1 copy of the *cfsA* genes (Table 7.1). These strains have different genetic background and the difference in CF production is possibly due to the fact that the putative *cfsB* gene(s) carried by pAL4404 might be less effective than that carried by pTiB6Tra^c, because strain Ach5(pTiAch5) is a Tra^{ie} strain and very inefficient in CF production (Chapters 2 and 3).

Strains A6(pTiA6; pBK129) and A6(pTiA6; pBK218) produce more than 68 ng of conjugation factor (per colony/24 h) in the presence of octopine (Table 7.1), and this is comparable to that produced by K588 (pTiB6Tra^c; pBK129) (Table 7.3). But, if no octopine is present in the minimal medium, these two strains produce a much lower level (14 - 15 ng) of CF (Table 7.1), which seems to be unproportional to their likely *cfsA* gene copy number. One possibility to explain this is that pTiA6 encodes a constitutively expressed repressor which partially inhibits the expression of *cfsA* genes contained by pBK129 and pBK218, and octopine can inactivate this repressor as proposed by Tempé et al. (1978). This possibility is further investigated in Chapter 8.

TABLE 7.3 CF overproduction by increasing copy number of *cfsA* locus in strain K588

Strain (plasmids)	CF production (ng)		Increase (times)	
	24 h	36 h	24 h	36 h
K588 (pTiB6Tra^{c})	17.3	21.68	-	-
K588 (pTiB6Tra^{c}; BK129)	74.99	105.18	4.3	4.9
K588 (pTiB6Tra^{c}; pLZT210)	66.98	101.09	3.9	4.7

CONCLUSION

Conjugation factor biosynthesis function is encoded by two (or more) different *cfs* loci on pTiB6Tra^{c}. The *cfsA* locus was subcloned and mapped in the Occ/Tra region of the octopine-type Ti plasmid by deletion analysis. The *cfsB* locus is likely contained within or near the Vir region of the Ti plasmid.

Conjugation factor production by strain K588 (pTiB6Tra^{c}) is increased by about 4 to 5 times when a *cfsA* clone carried by cosmid vector pVK102, a derivative of plasmid RK2 (which has 5 copies in *E. coli*), was introduced to that strain by mating. It appears likely that *cfsA* is a structural gene of the conjugation factor.

CHAPTER 8 MULTIPLE GENETIC REGULATION OF CONJUGATION FACTOR BIOSYNTHESIS IN *AGROBACTERIUM*

INTRODUCTION

Conjugation factor biosynthesis is encoded by two (or more) *cfs* loci on the Ti plasmid (Chapter 7). In wild type *A. tumefaciens* octopine strains, conjugal transfer of the Ti plasmid, CF production and octopine catabolism are all induced by octopine (Tempé et al, 1978; Klapwijk et al., 1978; Zhang and Kerr, 1991). Therefore, it is very likely that there is a single regulator controlling these three different biochemical and genetic functions. Based on the fact that the ocpoine catabolism constitutive strains also transfer Ti plasmid constitutively, Tempé et al. (1978) and Klapwijk and Schilperoort (1979) suggested that there is a repressor regulating both Ti plasmid conjugal transfer and octopine catabolism. But more recent molecular studies (Habeeb et al., 1991; Von Lintig et al., 1991) revealed that the transcription of the octopine catabolism operon of the Ti plasmids pTiA6 and pTiAch5 is activated by a positive regulator which is encoded by the *occR* gene. However, the role of its product, OccR in octopine-induced Ti plasmid conjugal transfer in octopine strains has not been determined.

This Chapter describes (1) isolation of Tra$^-$, Cfs$^-$ and Occ$^-$ mutants using transposon mutagenesis; (2) molecular cloning and subcloning of the principle genes for the regulation of conjugation factor biosynthesis and conjugal transfer of the Ti plasmid in octopine strains of *A. tumefaciens*; (3) the likely molecular regulatory mechanism of conjugation factor production in *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial Strains And Plasmids

A. tumefaciens strains A6 (pTiA6) and K749 have been described in Chapter 2; strains K588 (pTiB6Tra^{c}) and LBA4404 (pAL4404) were described in Chapter 7. *E. coli* strain HB101 and triparental mating helper plasmid pRK2013 were also described in Chapter 7. Transposon-mediated Tra^{-}, Cfs^{-} and Occ^{-} mutants are described in Tables 8.1 and 8.2. Cosmid clones pA6T140, pA6T211 and the *Hin*dIII deletion derivatives of pA6T140 are described in Fig.8.2.

Media And Chemicals

Media, growth conditions and chemicals have been described in Chapter 7.

Tn5 Insertional Mutagegesis Of Strain A6

The suicide plasmid pSUP1011 (Simon et al., 1983) in *E. coli* SM10 was used to introduce transposon Tn*5* insertions into the genome of *A. tumefaciens* octopine strain A6 by the protocol described by Garfinkel and Nester (1980), except that the bacterial suspensions were spread onto BM minimal plates containing kanamycin (100 µg/ml).

Identification of Tra^{-} Mutants and Determination Of Conjugal Transfer Efficiency

The replica plate mating method described in Chapter 2 was used with minor modification to identify Tn*5* mediated Tra^{-} mutants. In the standard replica plate mating method, the octopine concentration of 200 µg/litre was used in induction medium and the colonies were patched in a grid pattern, initially at about 1.2 cm apart. However, in order to minimise the diffusion effect of CF, which could "rescue" Cfs^{-} mutants (Chapter

3), the distance between adjacent colonies was increased to 2 cm. Tn*5* mediated Tra^- donors were identified by lack of transconjugant growth on the selective plates.

The drop mating method described in Chapter 2 was used to assay for Ti plasmid conjugal transfer efficiency.

Plasmid Purification And Isolation Of Total DNA Of Agrobacterium

Plasmid DNA was purified as described in Chapter 6. Total DNA of *Agrobacterium* was isolated as described by Ophel and Kerr (1990).

Construction Of Cosmid Libraries Of pTiA6 And Total DNA Of Strain A6

The same procedures described in Chapter 7 were used to construct cosmid libraries of pTiA6 and total DNA of strain A6.

DNA Probing

Colony hybridisation has been described in Chapter 7. Southern blotting was carried out as described by Maniatis et al. (1982).

Deletion Analysis Of Clone pA6T140

The method has been described in Chapter 7 for clone pBK129.

Construction Of Merodiploid Strains

Method for construction of merodiploid strains has been described in Chapter 7.

Conjugation Factor Activity Bioassay And Octopine Catabolism Determination

The CF diffusion plate bioassay using reporter strain NT1 (*traR*; *tra::lac*Z749) described in Chapter 7 was used without quantitative measurement. The bromothymolblue indicator medium for determination of octopine catabolism has been described (Chapter 7).

RESULTS AND DISCUSSION

8.1 Strategic Approach To Isolate Transposon-mediated Conjugation Factor Biosynthesis Deficient Mutants

Random Tn*5* insertion mutagenesis was used in an attempt to locate genes involved in CF biosynthesis, including structural and regulatory genes. Tn*5* is a very useful transposable element carrying a kanamycin resistance gene; it has the feature of random insertion (reviewed by Bruijn and Lupski, 1984). When inserted into a transcriptional unit, normal transcription will stop at the termination signal carried by Tn*5*, and the incomplete transcript cannot produce a functional product in most cases. The mutated gene can be readily cloned by selecting Km resistance or by using Tn*5* as a probe.

The Tn*5* mutagenesis experiments were performed before the CF bioassay strain NT1(*traR*; *tra::lacZ*749) was obtained from S. Farrand.

The CF activity bioassay described in Chapter 3 is not suitable for screening of Cfs$^-$ mutants from a large number of Tn*5* mutants. Screening for CF gene mutants was therefore based on their Tra$^-$ phenotype using the replica plate method described in Chapter 2. Since CF is an induction signal molecule in the Ti plasmid conjugal transfer system (Chapter 3 and Chapter 4), CF structural gene mutants should have a phenotype of Cfs$^-$Tra$^-$ and their Tra function should be complemented by adding CF to the induction medium. Mutation in regulatory genes controlling CF and other transfer functions should also produces a phenotype of Cfs$^-$Tra$^-$, and the Tra function of this type of mutants would not necessarily be complemented by external CF. This is based on the consideration that although CF can significantly enhance Ti plasmid conjugal transfer efficiency in the presence of a low concentration of octopine, it is not able to induce transfer by itself (Chapter 3). This suggests that besides those functions induced by CF, other gene products induced by octopine are also necessary for Ti plasmid conjugal transfer.

8.2 Transposon Insertional Mutagenesis To Identify Regions Of pTiA6 And Chromosomal DNA Of Strain A6 Required For Conjugal Transfer

Mutagenesis was accomplished by using the transposon carrier replicon pSUP1011 which contains Tn*5* inserted into the pSUP101, a mobilizable vector derived from the *E. coli* vector pACYC184 (Simon et al., 1983). When pSUP1011 is transferred to *Agrobacterium* from an *E. coli* mobilising strain, the plasmid is unable to replicate, but Tn*5* transposes at a low frequency from the plasmid before it is lost. Tn*5* mutants of *Agrobacterium* were selected on BM medium containing kanamycin because *E. coli* strains cannot grow on this minimal medium. To minimise the number of siblings, one plate containing 100-150 colonies was chosen from each of 30 independent matings. More than three thousand kanamycin-resistant *Agrobacterium* colonies were tested for Ti plasmid conjugal transfer. Transfer-deficient mutants were identified by their inability to generate octopine-utilising transconjugants when replica plate-mated with the recipient strain K749. All apparent transfer-deficient mutants were tested for utilisation of octopine because mutants with Tn*5* insertions inactivating octopine-catabolic (*occ*) genes would appear Tra$^-$ by this assay. This approach yielded 11 Ti plasmid conjugal transfer-deficient and 4 octopine-catabolism-deficient mutants (Table 8.1).

Conjugal transfer efficiency of the Tra$^-$ mutants was assayed quantitatively by the drop mating method (Zhang and Kerr, 1991; Chapter 2) at different octopine concentrations. Data in Table 8.1 show that all Tra$^-$ mutants have very low levels of Ti plasmid transfer when 200 mg/litre of octopine was used in the induction medium. But when the concentration of octopine was increased to 1200 mg/litre, some Tra$^-$ mutants produced a relatively high level of plasmid conjugal transfer.

TABLE 8.1 Transposon-mediated Tra⁻ and Occ⁻ mutants of strain A6*

Strain	Transfer efficiency in the presence of octopine		Octopine utilisation
	200 mg/L	1200mg/L	
M2	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	+
M28	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	+
M103	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	+
M111	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	+
M131	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	+
M61	0.35×10^{-6}	0.28×10^{-4}	+
M95	0.22×10^{-5}	0.47×10^{-4}	+
M104	$< 1 \times 10^{-8}$	0.63×10^{-5}	+
M107	0.31×10^{-5}	0.54×10^{-4}	+
M108	$< 1 \times 10^{-8}$	0.63×10^{-5}	+
M109	$< 1 \times 10^{-8}$	0.18×10^{-4}	+
M133	$< 1 \times 10^{-8}$	0.33×10^{-4}	+
M24	$< 1 \times 10^{-8}$	ND	-
M27	$< 1 \times 10^{-8}$	ND	-
M48	$< 1 \times 10^{-8}$	ND	-
M106	$< 1 \times 10^{-8}$	ND	-

* Abbreviations used: Tra⁻, transfer deficient; Occ⁻, octopine catabolism deficient; ND, not determined.

8.3 Identification Of Cfs$^-$ Mutants

All mutants listed in Table 8.1 were further tested for CF production (see Table 8.2). Surprisingly, none of the Tra$^-$ and Occ$^-$ mutants tested can produce CF. However, because the CF bioassay method used for this experiment is not very sensitive, a precise quantitative assay such as described in Chapter 7 is needed to draw a final conclusion. All mutants were then tested for conjugal transfer of their Ti plasmid in the presence of CF. Data in Table 8.2 show that 9 out of 16 mutants can transfer their Ti plasmid in the presence of CF.

Based on their phenotypes, 4 types of mutant have been temporarily defined: (1) Tra$^-$ group, including all Tra$^-$ mutants in which conjugal transfer efficiency cannot be enhanced by external CF; (2) Tra$^-$Cfs$^-$ group, including all Tra$^-$ mutants in which conjugal transfer efficiency is greatly enhanced by external CF; (3) Occ$^-$ group, including three Occ$^-$ mutants that have a positive CF response; (4) Occ$^-$Tra$^-$Cfs$^-$ group, that is strain M106 which shows a negative CF response.

As described above, the Occ$^-$ mutants has been divided into 2 classes. Those that respond to CF (i.e. restore Ti plasmid transfer) and those that don't. For those mutants responding to CF, it was noted that in the absence of external CF there was not any transconjugant colonies in the selective plates, however, if CF solution was added to the induction medium, some tiny transconjugants colonies appeared on the selective plates. The possible expaination is that in these three mutants the Tn*5* inserted into the *occ* genes encoding catabolic enzymes instead of into the octopine permease or *occ* regulatory gene; under this circustance, the mutants can't degrade octopine to provide a N source for the synthesis of CF, therefore, in this type of mutants adding CF restores conjugation. In the non-response mutant M106, it is possible that it may represent the inactivation of a common regulatory gene or alternatively, as octopine is the inducer of Occ, Tra and Cfs, mutation of an octopine permease would also generate the same Occ$^-$Tra$^-$Cfs$^-$ phenotypes.

TABLE 8.2 Identification of Cfs⁻ mutants in strain A6*

Strain	CF production	CF response†	Tn5 location	Classification
M2	ND	-	ND	Tra⁻
M28	ND	-	ND	Tra⁻
M95	ND	-	ND	Tra⁻
M111	-	-	pTi	Tra⁻
M131	-	-	pTi	Tra⁻
M104	-	-	Chr	Tra⁻
M61	-	+	ND	Tra⁻Cfs⁻
M107	-	+	pTi	Tra⁻Cfs⁻
M103	-	+	Chr	Tra⁻Cfs⁻
M108	-	+	Chr	Tra⁻Cfs⁻
M109	-	+	Chr	Tra⁻Cfs⁻
M133	ND	+	Chr	Tra⁻Cfs⁻
M24	-	+	ND	Occ⁻
M27	-	+	ND	Occ⁻
M48	-	+	Chr	Occ⁻
M106	-	-	pTi	Occ⁻Tra⁻Cfs⁻

* Abbreviation used: Chr, chromosomal DNA; pTi, Ti plasmid; Cfs⁻, CF synthesis deficient; the others refer to Table 8.1.

† CF response + means that conjugal transfer efficiency of Ti plasmid has been markedly enhanced by external CF.

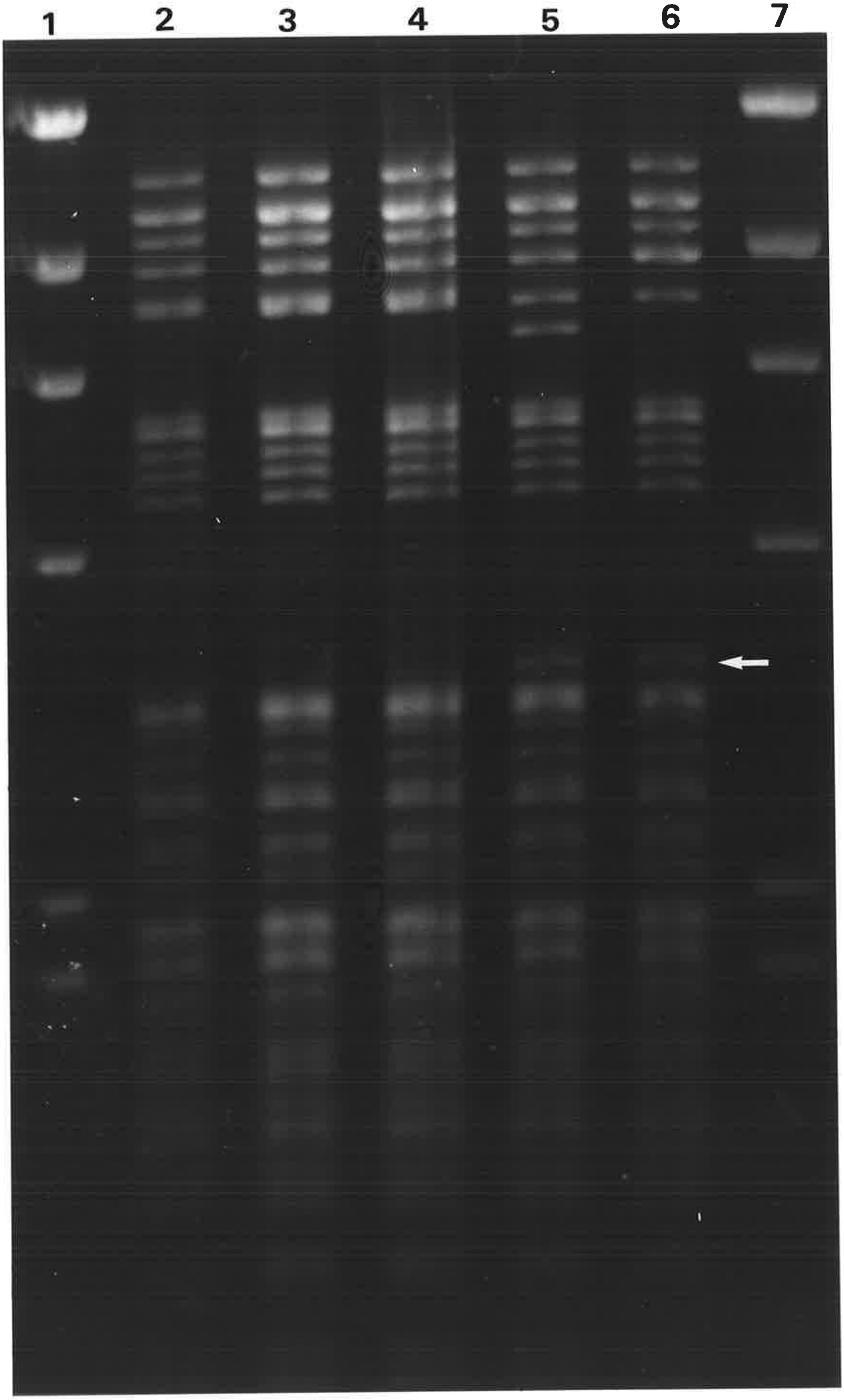
Further cloning and characterisation of the *cfs* genes was attempted. Experiments were conducted to see whether Tn*5* insertions in these Cfs$^-$ mutants are located on the Ti plasmid or on chromosomal DNA. Ti plasmids were purified from these mutants by the EtBr-phenol extraction procedure (Chapter 6) and digested by restriction endonuclease *Hin*dIII. After electrophoresis, mutants that have a Tn*5* insert in the Ti plasmid show a distinct 3.3 kb fragment (Fig 8.1). Among 12 mutants analysed, 6 of them were shown to have Tn*5* inserted in the Ti plasmid (see Table 8.2). The others probably have Tn*5* inserts in the chromosomal DNA since strain A6, the parental strain of these mutants, has only one plasmid (Chapter 6).

8.4 Cloning And Deletion Analysis Of A CF Biosynthesis Negative Regulatory Region

Tempé et al. (1978) and Klapwijk and Schilperoort (1979) suggested that there is a common repressor controlling Ti plasmid conjugal transfer and octopine catabolism. The results in Chapter 7 also indicate that pTiA6 might encode a constitutively expressed repressor. It was decided to try to locate regulatory genes in the Occ/Tra region (Fig. 7.3, Chapter 7) of a wild type Ti plasmid pTiA6, since regulatory genes are very likely to be in the same region as structural genes.

A cosmid library of wild type Ti plasmid pTiA6 was constructed and screened for the Occ/Tra region using the fragments adjacent to the Tn*5* insertion of M106 as a probe. Four clones (pA6T140; pA6T144; pA6T211; pA6T226) that were homologous to the probe were further analysed by restriction enzyme digestion and the results showed that pA6T144, pA6T211 and pA6T226 contain the same clonal insert. Therefore, pA6T140 and pA6T211 were mated into *Agrobacterium* strain K588(pTiB6Tra^c) which is a CF biosynthesis constitutive strain. The transconjugants were tested for CF production. The results (Fig. 8.2; Fig. 8.3) show that K588 (pTiB6Tra^c; pA6T140) no longer produces CF constitutively but produces CF only after

Fig.8.1 *Hin*dIII restrition enzyme digestion of Ti plasmids isolated from Tra$^-$ and Cfs$^-$ mutants to identify Tn*5* insertions. Arrows indicate the 3.3 kb Tn*5* fragment generated by *Hin*dIII digestion. Lane 1, pTiA6; lane 2, pTiM103; lane 3, pTiM109; lane 4, pTiM111; lane 5, pTiM131.


1
2
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induction by octopine, indicating pA6T140 contains a repressor which is inactivated by octopine.

To locate the repressor region, different sized *Hin*dIII deletion clones of pA6T140 were prepared, mated into a CF synthesis constitutive (Cfs^{C}) strain K588 (pTiB6Tra^{C}) and assayed for CF production with and without octopine induction. As shown in Fig.8.2 and Fig 8.3, all deletion clones containing *Hin*dIII fragment No.1 of cosmid clone pA6T140, except for cosmid clone pA6T211, repress the Cfs^{C} phenotype in strain K588(pTiB6Tra^{C}) in the medium without octopine. As was expected, CF production in these recombinant strains was induced by octopine.

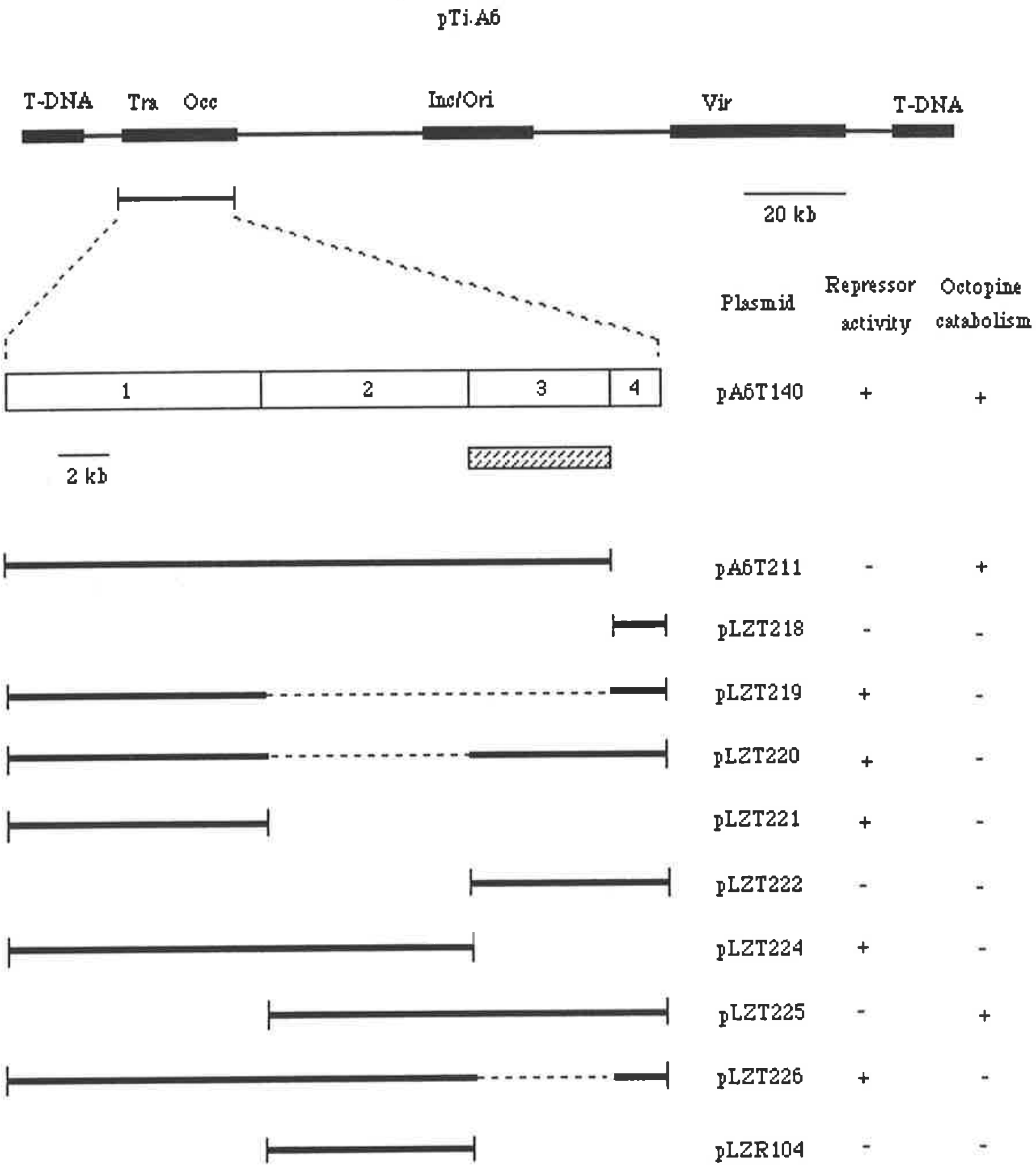
It is surprising that cosmid clone pA6T211 did not show any repressor activity (Fig.8.2 and Fig. 8.3). DNA hybridisation data indicate that pA6T211 contains all *Hin*dIII fragments except fragment 4 of cosmid clone pA6T140. The experiment described in the next section indicates that pA6T211 might contain an inactivated repressor gene.

8.5 Identification Of A Putative CF Biosynthesis Positive Regulatory Region

Since a single insertional mutation using Tn*5* can generate an Occ^{-}Cfs^{-}Tra^{-} phenotype (strain M106, Tables 8.1 and 8.2), and the Tra function cannot be rescued by adding CF, it seems likely that there is a common positive regulatory gene, although an alternative explanation is possible (refer to section 8.3).

Cosmid clones pA6T140 , pA6T211 that are homologous to the M106 probe, and deletion derivatives of pA6T140 were mated into LBA4404 (pAL4404). The recombinant strains were assayed for CF biosynthesis (Cfs) activity. Fig.8.4 shows that only strain LBA4404 (pAL4404; pA6T140) can produce CF in the octopine induction medium, while strains LBA4404 (pAL4404; pA6T211), LBA4404 (pAL4404; pLZT221), LBA4404 (pAL4404; pLZT224), and LBA4404 (pAL4404; pLZT225) cannot produce CF. These data indicate that the *Hin*dIII fragments 3 and 4 of pA6T140

Fig.8.2 Deletion analysis of the CF biosynthesis negative regulatory region. The physical map (*Hin*dIII) of cosmid clone pA6T140 was constructed by deletion analysis. *Hin*dIII fragments of pA6T140 were numbered according to their sizes. The hatched box under the map of pA6T140 indicates the region homologues to M106 probe. pA6T211 has been described. pLZR104 was constructed by cloning of the *Hin*dIII fragment No. 2 of pA6T140 into cosmid vector pVK102. Deletions of pA6T140 were prepared as described. The plasmids were mated into CF biosynthesis constitutive strain K588(pTiB6Tra^c), and assayed to see if they inhibit CF biosynthesis in the absence of octopine.



Plasmid	Repressor activity	Octopine catabolism
pA6T140	+	+
pA6T211	-	+
pLZT218	-	-
pLZT219	+	-
pLZT220	+	-
pLZT221	+	-
pLZT222	-	-
pLZT224	+	-
pLZT225	-	+
pLZT226	+	-
pLZR104	-	-

Fig.8.3 Repressor inhibits CF biosynthesis in constitutive strain K588(pTiB6Tra^{c}). Deletion clones of pT140 have been described in Fig.8.1. CF bioassay has been described. CF producing strains were patched onto the squares on the right side of the plates, and the CF bioassay strain was spotted at regular distances onto the left side of the squares. Double lines indicate that a slice of agar has been removed. The blue colonies (dark as shown in the picture) in the plates indicate that *tra::lacZ*749 fusion genes contained in the bioassay strain have been induced by diffusible CF. Right: without octopine; Left: plus octopine. Strains used: A, K588(pTiB6Tra^{c}); B, K588(pTiB6Tra^{c}; pA6T140); C, K588(pTiB6Tra^{c}; pA6T211); D, K588(pTiB6Tra^{c}; pLZT218); E, K588(pTiB6Tra^{c}; pLZT222); F, K588(pTiB6Tra^{c}; pLZT224); G, K588(pTiB6Tra^{c}; pLZT225); H, K588(pTiB6Tra^{c}; pLZT219).

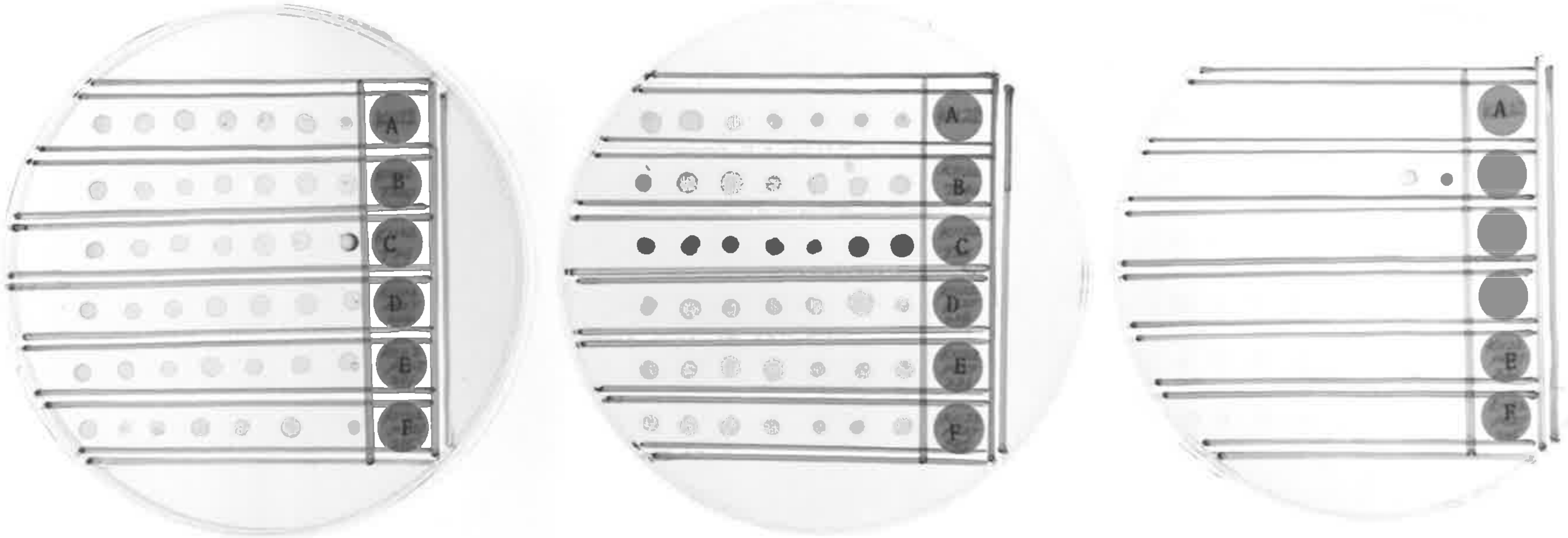

A
B
C
D


A
B
C
D


E
F
G
H


E
F
G
H

Fig.8.4 The Occ region may contain a CF biosynthesis positive regulatory gene. Deletion clones have been described in Fig.8.1. The CF bioassay was as described in Fig.8.2. Double lines indicate that a slice of agar has been removed. From right to left: octopine (200 mg/litre) induction medium; $(NH_4)_2SO_4$ (200 mg/litre) replaced octopine; Proline (200 mg/litre) replaced octopine. Strains used: A, LBA4404 (pAL4404); B, LBA4404 (pAL4404; pA6T140); C, LBA4404 (pAL4404; pA6T211); D, LBA4404 (pAL4404; pLZT224); E, LBA4404 (pAL4404, pLZT221); F, LBA4404 (pAL4404; pLZT225).


A
B
C
D
E
F
A
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D
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A
E
F

are essential but not sufficient for CF biosynthesis. The *Hin*dIII fragments 2, 3 and 4 of pA6T140 encode Occ function (Fig.8.2). In strain Ach5, a positive regulator OccR was found to be encoded by a 2 kb *Hin*dIII fragment (Von Lintig et al., 1991) which is the same size as fragment 4 of pA6T140 (Fig.8.2). The OccR gene from pTi A6 has also been sequenced and shown to be exactly the same as the *occR gene* of pTiAch5 (Habeeb et al., 1991), but the *Hin*dIII physical map of that region was not presented making comparison with pA6T140 difficult. A region encoding a putative periplasmic octopine permease was also characterised (Valdivia et al., 1991), which should be located in *Hin*dIII fragment 3 of pA6T140 if *Hin*dIII fragment 4 of pA6T140 encodes OccR. The above data suggest that CF biosynthesis is positively controlled by the Occ region.

If *Hin*dIII fragment 4 of pA6T140 encodes OccR, the positive regulator of octopine catabolism, it is logical that recombinant strain LBA4404 (pAL4404; pA6T211) cannot produce CF in the octopine induction medium since it does not have an *occR* gene, but there is no obvious explanation why this strain produces CF constitutively (Fig.8.4). One possibility is that the repressor gene contained in *Hin*dIII fragment 1 of pA6T211 was somehow inactivated. This seems likely because PA6T211 did not show any repressor activity (Fig.8.3).

8.6 Cloning Of A CF Biosynthesis Regulatory Locus From Chromosomal DNA of Strain A6

Transposon mutagenesis experiments (Table 8.2) show that *Agrobacterium* chromosomal genes are also involved in CF biosynthesis. Of particular interest is strain M103, in which a single insertional mutation results in a complete Tra$^-$Cfs$^-$ phenotype even at elevated concentration of octopine (Table 8.1), but the Tra$^-$ phenotype can be "rescued" by adding CF to the induction medium (Table 8.2), suggesting that CF biosynthesis is totally inhibited by the Tn*5* insertion. Molecular studies were therefore performed to distinguish if the mutated gene (*cfs*) is a structural or regulatory gene.

Total DNA of *A. tumefaciens* strain A6 was partially digested with *Hind*III and cloned into the broad host-range cosmid vector pVK102 (Knauf and Nester, 1982); the clone bank was probed using the adjacent fragments to the Tn*5* insertion of CF$^-$ mutant M103 as a probe. In this way, six clones were isolated. Digestion with restriction enzymes (*Hind*III and *Eco*RI) indicate that five clones have the same DNA insert (pLZN1426, Fig.8.5) and one is different (pLZN828, Fig.8.5). These two plasmids were transferred, by triparental mating, into the Cfs$^-$ mutant M103. Transconjugant colonies were assayed for CF production and Ti plasmid conjugal transfer. Neither of the clones can complement the Cfs$^-$ and Tra$^-$ mutant even in the presence of 1200 mg/litre octopine (Table 8.4). However, when the octopine induction medium was supplied with 10 g/litre of mannitol, transconjugants appeared (Table 8.4). A higher rate of plasmid transfer also occurred when EDTA were added to the induction medium (Table 8.4). However, even in the presence of mannitol in the induction medium, these two clones fail to complement the Cfs$^-$ phenotype of strain M103. There is no obvious explanation why complementation is so inefficient.

To test whether the cloned region is a structural gene or regulatory gene, its presence in other *Agrobacterium* strains was determined. DNA hybridisation experiments (Fig.8.6) indicate that the M103 probe hybridises with total DNA of the octopine strain A6, total DNA of nopaline strain C58, and pTiC58; but not with pTiB6Tra^C, total DNA of K749, total DNA of nopaline strain T37 and pTiT37. Since strains K749(pTiB6Tra^C) and T37 can produce CF (Zhang and Kerr, 1991; Chapters 3 and 7), but don't have this gene, it cannot be a structural gene, suggesting it is a regulatory gene. Mutation of this gene by Tn*5* insertion has no effect on normal octopine catabolism (see strain M103, Table 8.1), this gene is therefore likely only involved in the regulation of the CF biosynthesis and designted a conjugation factor regulatory (*cfr)* gene.

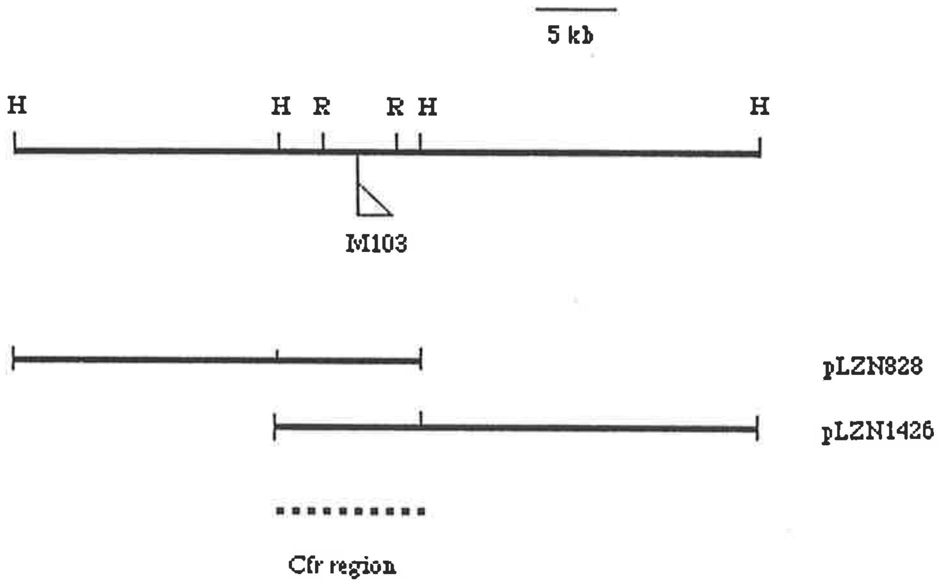


Fig.8.5 Physical maps of the Cfr region and relevant plasmids. Tn*5* insertion ΩM103 and its corresponding strain M103 have been described in the text. The map was constructed based on restriction enzyme digestion patterns and DNA hybridisation.

Table 8.4 Complementation analysis of Tn*5*-mediated Tra^-Cfs^- mutant M103 of strain A6*

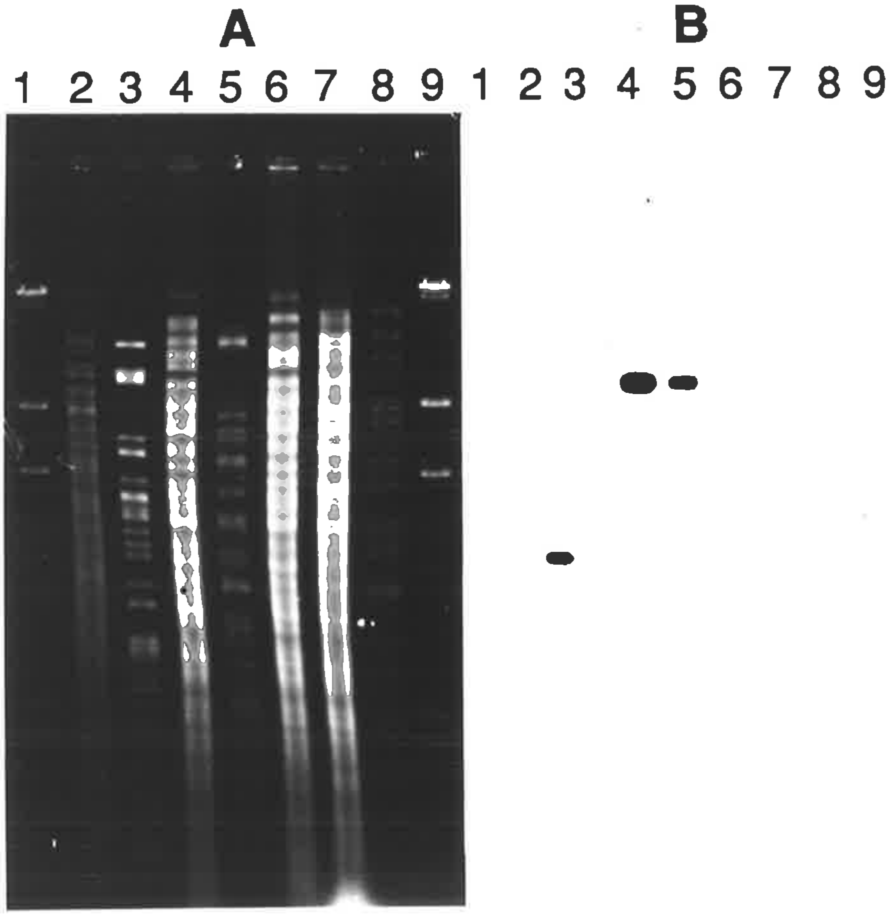
Strain and plasmid	Chemicals added (mM)	Transfer efficiency†
M103	-	$< 1 \times 10^{-8}$
M103	mannitol (55)	$< 1 \times 10^{-8}$
M103	EDTA (0.2)	$< 1 \times 10^{-8}$
M103 (pLZN828)	-	$< 1 \times 10^{-8}$
M103 (pLZN828)	mannitol (55)	0.63×10^{-6}
M103 (pLZN828)	EDTA (0.2)	0.27×10^{-5}
M103 (pLZN1426)	-	$< 1 \times 10^{-8}$
M103 (pLZN1426)	mannitol (55)	0.33×10^{-6}
M103 (pLZN1426)	EDTA (0.2)	0.48×10^{-5}

* Octopine concentration used in the induction medium is 1200 mg/litre.

† Conjugal transfer efficiency is expressed as transconjugants/per donor cell.

The experiments described in this Chapter identify three CF biosynthesis regulatory loci in *Agrobacterium* octopine strain A6; one encodes a repressor while the other two are very likely positive regulatory genes. But, studies such as reporter gene assays are needed to further investigate how these three regulatory components cooperate in the regulation of CF biosynthesis and conjugal transfer of the Ti plasmid.

Fig.8.6 DNA hybridisation to identify regions homologous to the *cfr* probe in different *Agrobacterium* strains. A: DNA *Eco*R1 digestion patterns; B: Southern blotting patterns with the *cfr* probe cloned from strain M103. Symbols: 1 and 9 are *Hin*dIII digested λ-DNA; 2, Total DNA of octopine strain A6; 3, pTiB6Tra^{c}; 4. Total DNA of nopaline strain C58; 5, pTiC58; 6, Total DNA of plasmidless nopaline strain K749; 7, Total DNA of nopaline strain T37; 8, pTiT37.


A
B
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9

CONCLUSION

A total of 12 Tra$^-$ (Ti plasmid conjugal transfer deficient) and 4 Occ$^-$ (octopine catabolism deficient) mutants of *A. tumefaciens* octopine strain A6 were isolated following Tn*5* mutagenesis. Both of these types of mutants can be divided into 2 groups based on whether their Tra$^-$ phenotype can or cannot be complemented by external CF (conjugation factor). Those Tra$^-$ mutants whose Tra$^-$ phenotype can be complemented by external CF are classified as Cfs$^-$ mutants. Of the Occ$^-$ type mutants, only strain M106 whose Tra$^-$ phenotype cannot be complemented by external CF possibly contains a mutated common regulatory gene of Occ, Tra and Cfs, because both CF and octopine induced Tra function are known to be needed in Ti plasmid conjugal transfer (Zhang and Kerr, 1991; Chapter 3).

By using the fragments adjacent to the Tn*5* insertions of relevant Cfs$^-$ mutants as hybridisation probes, two cosmid banks, one from pTiA6 and one from total DNA of strain A6 were screened. Three CF biosynthesis regulatory loci have been identified and subcloned using deletion and complementation analysis. Two were located in the Occ/Tra region of pTiA6 and one in the chromosomal DNA of strain A6.

It is very likely that the *occR*, the octopine catabolism regulatory gene (Habeeb et al., 1991; Von Lintig et al., 1991), is a common regulatory gene, positively controlling Occ, Tra and Cfs functions. Tn*5* insertional mutagenesis in the Occ region of pTi A6 produced an Occ$^-$Tra$^-$Cfs$^-$ phenotype. When the Occ region was deleted from Cfs clone pA6T140, it lost its ability to produce CF in the background of strain LBA4404 (pAL4404).

CF biosynthesis is also negatively regulated by a repressor encoded by cosmid clone pA6T140. The repressor region was located within in a 10 kb *Hin*dIII fragment adjacent to the Occ genes. It represses CF production in strain K588 (pTiB6Tra^{c}), a CF biosynthesis constitutive mutant, when it mated into this strain. This repression is abolished in the presence of octopine.

CF biosynthesis is also regulated by a gene, named *cfr*, located in the chromosomal DNA of strain A6. Tn*5* insertion in this region causes a Cfs^{-}Tra^{-}Occ^{+} phenotype, but the Tra^{-} phenotype can be rescued by external CF. Therefore, *cfr* is only related to CF biosynthesis. This gene encodes a regulatory function, because some strains do not have this gene can produce CF. Two cosmid clones containing the Cfr region have been isolated and tested for complementation of Tra^{-} and Cfs^{-} phenotypes in the Cfs^{-}Tra^{-} mutant M103, but no complementation has been observed under normal assay conditions. However, a low level of complementation was observed in the presence of oxygen radical scavengers mannitol or EDTA in the octopine induction medium. This suggest *cfr* gene may be regulated by oxygen radicals.

It should be emphasized that these genetic studies are preliminary and much remains to be done to elucidate the detailed regulation mechanism of conjugation factor biosynthesis.

CHAPTER 9 GENERAL DISCUSSION

9.1 A Novel Conjugation Factor

Previous work has shown that Ti plasmid conjugal transfer in *A. tumefaciens* is induced by conjugal opines (Petit et al., 1978; Klapwijk et al., 1978; Tempé et al., 1978). But the molecular and biochemical mechanisms of this opine induced Ti plasmid conjugal transfer remained unknown. The investigations described in this thesis first demonstrate that the conjugal opine, octopine, induces a novel diffusible conjugation factor (Chapter 3) which then turns on *tra* genes to initiate the Ti plasmid conjugal transfer process (Chapter 4).

Octopine strains of *A. tumefaciens* can be placed in two distinct groups, viz, Tra^{e} (transfer efficient) and Tra^{ie} (transfer inefficient), based on their conjugal transfer efficiency. This distinction is also apparent in their response to different concentrations of octopine and in the time required for induction (Chapter 2). A conjugation factor (CF) was first observed to be produced by some Tra^{e} strains and it can significantly enhance the Ti plasmid conjugal transfer efficiency of Tra^{ie} strains (Chapter 3). Transfer constitutive (Tra^{c}) mutants can produce conjugation factor constitutively, Tra^{e} strains can produce it after induction by low octopine concentrations, whereas Tra^{ie} strains need longer induction time or higher octopine concentration for conjugation factor production (Chapter 3). Molecular genetic studies further confirm the important role played by CF in bacterial conjugation and Ti plasmid transfer. Transposon mediated Cfs^{-} (conjugation factor synthesis deficient) strains fail to transfer their Ti plasmid unless supplied with conjugation factor in the induction medium (Chapter 8, Chapter 4). The present data suggest that Ti plasmid conjugal transfer is mediated by two levels of regulation. A plant tumour produced conjugal opine which is the environmental signal, induces the synthesis of CF in *Agrobacterium* donor cells; CF behaves as a secondary messenger, transmitting the environmental information to *tra* genes. It appears to be the first example of a second messenger molecule in a bacterial conjugation system.

Conjugation factor was purified and identified as *N*-ß-oxo-octanoyl-L-homoserine lactone by mass spectrometry, infrared spectroscopy, nuclear magnetic resonance spectroscopy, and was verified by the synthesis of *N*-ß-oxo-octanoyl-L-homoserine lactone. The pure synthetic conjugation factor gave the characteristic IR, NMR, mass spectra and specified biological activity (Chapter 4). The chemical structure of *N*-ß-oxo-octanoyl-L-homoserine lactone (refer to Fig.4.9) explains the initial observations that conjugation factor is diffusible, amphiphilic, and easily denatured by alkaline conditions (Zhang and Kerr, 1991; Chapter 3; Chapter 4). The molecular weight of the conjugation factor is only 241; its 8 carbon side chain is the basis of its lipophilic properties, whereas its homoserine lactone moiety and the ketone and amide groups determine the hydrophilic properties (Chapter 4); the ester-like properties of homoserine lactone make it react readily with alkaline bases to form a less reactive functional group amide. As discussed in section 9.2, the structure of the lactone ring of CF is important for its biological activity.

9.2. CF-Like Molecules May Be A Group Of Conserved Signal Molecules In Microorganisms

Surprisingly, the chemical structure of conjugation factor is very similar to that of the *lux* autoinducer, *N*-ß-oxo-hexanoyl-L-homoserine lactone, in a very dissimilar bacterium *Vibrio fischeri*. (Eberhard et al., 1981). This *lux* autoinducer has also been recently reported to regulate carbapenem antibiotic biosynthesis in *Erwinia carotovora* (Bainton et al., 1992). Another N-acyl amide of homoserine lactone, *N*-ß-(hydroxy-butanoyl)-L-homoserine lactone, was found to be the autoinducer of luminescence in *V. harveyi* (Cao and Meighen, 1989). The *N*-hexanoyl amide of homoserine lactone has also been detected in culture filtrates of *Agrobacterium* with very low *tra* gene inducing activity (Chapter 4). The above data suggest that *N*-acyl homoserine lactones may be a group of highly specific and widely conserved signals for bacterial gene regulation, and that their functions are very likely determined by the length and nature of their lipophilic acyl chain.

By synthesis of a series of conjugation factor derivatives, it has been demonstrated that the length and nature (saturated or unsaturated) of the *N*-acyl side chain of conjugation factor is very important in its *tra* gene inducing ability. Increasing or decreasing 2 carbons in its side chain causes a decrease in its *tra* gene induction activity by four orders of magnitude or more. Its saturated analogues have shown little biological activity (Chapter 4).

Besides the *N*-acyl side chain, the lactone structure in the homoserine moiety of CF is also very important in its *tra* gene induction activity. As discussed in section 9.1, its lactone ring is opened in the presence of alkaline bases and an amide is produced. No biological activity was detected for this alkaline-treated conjugation factor (Chapter 3). As expected, its biological activity returns after its aqueous solution is neutralised by HCl.

A methyl ester of the conjugation factor was detected in the *Agrobacterium* culture filtrate and proved to be a methanolic-solvent transesterification artifact. But the quantitative bioassay data indicate that its biological activity is almost identical to that of conjugation factor (Chapter 4). Therefore , it seems very likely that there is an enzyme in *Agrobacterium* which can efficiently lactonise the methyl ester of conjugation factor.

9.3 CF Biosynthesis Is Encoded By Two Or More Loci In The Ti Plasmid

The biosynthesis of conjugation factor is clearly Ti plasmid dependent. When the CF producing strain K608 was cured of its Ti plasmid, it lost CF production ability, and conversely, strain K749 which is a plasmidless strain gained this ability after it received the Ti plasmid from strain K608 (Chapter 3).

CF is probably a common factor among different opine strains. In addition to octopine strains, nopaline Tra^{c} and Tra^{ci} strains K323 and T37 are able to produce CF or a very similar compound (Chapter 3). Another nopaline strain, C58 was also found to produce a conjugation factor like molecule (Piper et al., 1993).

Based on the facts that both octopine and nopaline strains can produce conjugation factor or a similar compound, we infer that *cfs* (conjugation factor synthesis) genes are contained in the conserved common regions of octopine and nopaline type Ti plasmids. There are four regions, viz, T-DNA, Vir, Ori/Inc and Occ/Tra (or Acc/Tra in nopaline type Ti plasmid), that are commonly conserved in these two types of Ti plasmid (Engler et al., 1981). The experiments described in Chapter 7 demonstrate that the *cfsA* locus is located within the Occ/Tra region. But the *cfsA* locus is only one of the loci required for CF biosynthesis; it needs to cooperate with a second locus carried by plasmid pAL4404 for CF production. Plasmid pAL4404 is a Ti plasmid deletion mutant with the T-DNA and Occ/Tra regions being deleted, and it therefore contains only Vir and Ori/Inc of the two common regions. It was originally used to provide Vir functions in binary vectors for plant transformation (Ooms et al., 1982; Hoekema et al., 1983). It seems unlikely that the Ori/Inc region contains a second *cfs* locus, because *Agrobacterium* strains carrying Ori/Inc clones of different origin did not show any Cfs activity in the presence of a *cfsA* clone (Chapter 7). Therefore, it would seem likely that the the second *cfs* locus is located within or near the Vir region.

Reports from two laboratories seem to support this possibility. Mutation of the *virA*, *virB*, *virC*, and *virG* genes of octopine type Ti plasmid pTiR10 was found to cause a 100- to 10,000-fold decrease in the frequency of conjugal transfer of this plasmid between *Agrobacterium* cells (Gelvin and Habeck, 1990); while in the nopaline type Ti plasmid pTiC58, mutations in *virA*, *virG*, *5'virB* and *virE* had a marked effect on plasmid conjugal transfer (Steck and Kado, 1990). However, it is also noted that the effect of mutation of *vir* genes on Ti plasmid conjugal transfer is not absolute. In the case of an octopine type Ti plasmid, it occurred only during early stages (18 to 24 h) of induction by octopine (Gelvin and Habeck, 1990). Also, transfer frequencies of pTiC58 derivatives containing mutated *vir* genes were all higher than 0.7×10^{-3} (Steck and

Kado, 1990). But, this phenomenon could be due to the fact that, besides conjugation factor, *Agrobacterium* also synthesizes *N*-hexanoyl amide of homoserine lactone (and perhaps other unidentified *N*-acyl amides of homoserine lactone) which has a low level of *tra* gene inducing ability (Chapter 4).

9.4 CF Biosynthesis Is Positively And Negatively Regulated In A. tumefaciens

It was first proposed by Tempé et al. (1978) that there might be a common repressor which blocks common operator sites in the promoter region of the Occ and Tra loci and octopine can derepress the operon by binding to the repressor, based on their observation that constitutive octopine catabolic (Occ^c) mutants are also transfer constitutive (Tra^c). However, a single insertion mutation can produce the phenotype Occ$^-$Tra$^-$ (Klapwijk et al., 1978; Klapwijk and Schilperoort, 1979), and therefore, a positive control gene might also be involved in the regulation of Ti plasmid conjugal transfer. Two recent molecular biological studies indicate that *occ* genes are regulated by a positive regulator, OccR (Lintig et al., 1991; Habeeb et al., 1991). But the role of OccR in Ti plasmid conjugal transfer and CF biosynthesis has not been established.

It has been shown in Chapter 3 that all opines tested (norooctopinic acid, homooctopine, agropine, nopaline and allo-nopaline) were able to induce strain K608 (pTiB6::Tn*5*, Tra^{ci}) to produce CF although their effect varied. Imino acids L-proline and D-proline could also induce CF production. But the amino acid, L-arginine, a substituent of octopine (Ménagé and Morel, 1964), had no effect on CF induction. Results suggest that the imino group might be the basic structure needed for CF induction in strain K608. However, CF production in the wild type strain A6 can be induced by octopine but not by L-Proline (Chapter 8). Strain K608 is a conjugal transfer partially constitutive and partially inducible (Tra^{ci}) mutant. It may be possible that it has a minor mutation in its regulatory gene which produces the Tra^{ci} phenotype and also changes its specificity for gene inducers. A similar case has been reported for nopaline which is not a conjugal opine, but can induce transfer of the Ti plasmid in strain T37 (Petit et al., 1978) which is also a Tra^{ci} mutant (refer to Table 3.1 in Chapter 3). These facts suggest that octopine is the "natural" inducer of CF biosynthesis function.

The data presented in Chapter 8 demonstrate that conjugation factor production is positively regulated by genes in the Occ region. When the Occ region was deleted from the *cfsA* cosmid clone pA6T140, it lost Cfs activity in the background of LBA4404 (pAL4404). It is likely that pA6T140 contains all *occ* genes, including *occR*, since this plasmid encodes a normal octopine catabolism function. It has been noted that the other *occ* genes cannot transcribe if the *occR* gene is deleted from the Occ region (Habeeb et al., 1991). Therefore, there seems to be little doubt that OccR also positively regulates CF biosynthesis and Ti plasmid conjugal transfer.

Data in Chapter 8 indicate that conjugation factor biosynthesis is also negatively regulated. The repressor is encoded by a 10 kb fragment of pTiA6 in the Occ/Tra region, but it is separated from occR (assuming it is located in *Hin*dIII fragment 4 of pA6T140) by a region of at least 13 kb. The Cfs^{c} mutant K588 (pTiB6Tra^{c}) resumes its wild type character after obtaining a plasmid containing this region, i.e., it cannot produce CF unless induced by octopine. However, it has not yet been determined whether this repressor also controls octopine catabolism.

A chromosomal locus named Cfr in octopine strain A6 has also been found to be involved in CF biosynthesis (Chapter 8). It seems to be a regulatory gene because those Cfs^{c} strains without this locus (as indicated by DNA hybridisation data using *cfr* probe) can produce conjugation factor constitutively. Tn*5* insertion in the Cfr locus of strain A6 produces a phenotype of Cfs^{-}Tra^{-}, but Occ function is not affected. It is therefore likely that the *cfr* gene is only involved in the positive regulation of conjugation factor biosynthesis. However, it is not known why in *trans* complementation of *cfr* insertional mutant by *cfr* cosmid clones pLZN828 and pLZN1426 is so inefficient. It seems unlikely that pLZN828 and pLZN1426 each contains only a part of the Cfr region, since a low level of complementation has been detected in the presence of oxygen radical scavengers such as mannitol and EDTA. In nopaline strain C58, it has also been noted that a Tra$^-$ mutant mediated by Tn*5* insertion is not complementable in *trans* (von Bodman et al., 1989).

Mannitol and EDTA are oxygen radical scavenger (Gutteridge et al., 1979; Gutteridge, 1984; Halliwell and Gutteridge, 1986) and can significantly counteract the inhibition of CF biosynthesis by oxygen radicals in shaking liquid culture (Chapter 5). It would be interesting to see if it is this *cfr* gene which is regulated by oxygens radical.

The genetic regulation of CF biosynthesis and Ti plasmid conjugal transfer in octopine type strains is probably different from that in nopaline type strains. It was found that in nopaline type strain C58, both Acc (agrocinopine catabolism) and Tra functions are coordinately controlled by a single repressor, AccR (von Bodman et al., 1992); this was referred to as the first level of regulation. Whereas in octopine strains, as descussed above, the Occ, Cfs and Tra functions are very likely coordinately regulated by OccR, a transcriptional activator (Habeeb et al., 1991; Von Lintig et al., 1991). The second level of regulation of Ti plasmid conjugal transfer in nopaline strains operates through a positive activator, TraR which needs to cooperate with CF for induction of *tra* genes (Piper, 1993). It has not yet been determined if TraR also regulates CF biosynthesis. Also, it is not known whether the Cfs negative repressor of octopine strains described in Chapter 8 is equivalent to TraR of nopaline strains. But the available evidence seems to indicate that these two types of strain might have conserved structural Tra functions, but have a different regulatory apparatus.

9.5 Oxygen Radicals Regulating CF Biosynthesis

Initially, it was noted that although CF production by wild type octopine strains such as A6 can be observed in solid agar plates, CF activity was not detected in liquid culture filtrate. The experiments described in Chapter 5 show that this inhibitory effect is due to oxygen radicals. In living organisms, oxygen radicals can be produced by the incomplete reduction of oxygen during respiration (Storz et al., 1990). There are different species of oxygen radical including superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxy radical ($HO\bullet$). But the most reactive species is the hydroxy radical, $HO\bullet$ (Halliwell and Gutteridge, 1986; reviewed by Sutherland, 1991). It is generally proposed that hydroxy radicals could result from a Fenton-type reaction (Czapski, 1984): $H_2O_2 + Fe^{2+} \rightarrow HO\bullet + HO^- + Fe^{3+}$. This explains why catalase, hydroxy radical scavengers , and EDTA (iron chelator) all show significant counter-inhibition effects when added to shaking liquid cultures of *Agrobacterium* (Chapter 5).

It is noted that bacterial growth and octopine catabolism are not much influenced by the oxygen radicals produced in shaking liquid culture, whereas CF biosynthesis in wild type octopine strains has been significantly repressed. However, for CF production constitutive strains, no inhibition has been observed in shaking liquid culture. It seems likely that there is a regulatory step in CF biosynthesis which is very sensitive to oxygen radicals.

9.6 Mode Of Action - Induction of tra Gene Expression by Conjugation Factor

A recent report from Farrand's group (Piper et al., 1993) on the nopaline plasmid pTiC58 shows that conjugal transfer is directly regulated by a transcriptional activator, TraR which requires conjugation factor to activate *tra* gene expression. This is very similar to the induction of *lux* genes in *V. fischeri*. The expression of *lux* genes (*luxR* and *luxICDABEG*) requires autoinducer (*N*-ß-oxo-hexanoyl homoserine lactone) and LuxR, a transcriptional activator (Engebrecht et al., 1983). The two regulatory systems are clearly related. Not only is CF structurally very similar to the *lux* autoinducer (Zhang et al., 1993; Chapter 4), the transcriptional activator TraR encoded by pTiC58 also exhibits 19% amino acid identity and 62% amino acid similarity over a 234 residue overlap with LuxR (Piper et al., 1993).

However, it is not yet known how conjugation factor induces the expression of *tra* genes in octopine strains. Data in Chapter 3 show that in octopine strains, conjugation factor is a key factor affecting Ti plasmid conjugal transfer efficiency, but is not sufficient by itself to induce conjugal transfer of Ti plasmid; the presence of octopine is needed. It seems likely that octopine strains are similar to nopaline strains in which the *traR* product is needed for CF function (Piper et al., 1993; see section 9.4), that is a *tra* gene product induced by octopine is needed to cooperate with conjugation factor to initiate Ti plasmid transfer. However, as discussed in section 9.4, the regulation mechanism of Ti plasmid conjugal transfer and CF biosynthesis in octopine and nopaline strains seems to be different. Much remains to be done to reveal the details of the regulatory mechanism.

9.7 Relationship Between Ti Plasmid Conjugal Transfer And T-DNA Transfer

Data presented in Chapter 2 suggest that Ti plasmid conjugal transfer between bacteria and T-DNA transfer from bacterium to plant are very likely to have totally different mechanisms. An important observation in this regard, was that efficient Ti plasmid transfer occurred at 34°C. Even at 37°C conjugal transfer probably occurs because transconjugants appeared two days after transfer from 37°C to 28°C. At 28°C, transconjugants normally appeared after 4 days. It seems likely that Ti plasmid transfer occurred at 37°C but transconjugants did not appear on selective plates because *A. tumefaciens* cannot grow at 37°C. However, on transfer to 28°C, they developed rapidly. The result confirms the observation of Tempé *et al* (1977) that Ti plasmid conjugal transfer of strain B6S3 did not occur at temperatures above 30°C. However, it is an exception and most strains can transfer the Ti plasmid at 34°C. Induction of *vir* genes, on the other hand, is inhibited by a temperature above 30° C (Alt-Mörbe et al., 1988; Alt-Mörbe et al., 1989). Even for strain B6S3, when CF was added to the induction medium, the Ti plasmid transfer efficiencies of strain B6S3 were 0.93×10^{-3} and 0.55×10^{-4} at 32°C and 34°C respectively (Chapter 3). These data suggest that there is a thermosensitive step involved in CF production by strain B6S3, or possibly, the induction or derepression of *tra* genes of strain B6S3 needs higher concentration of CF at elevated temperatures. Strain B6S3 is a phage-sensitive isolate from U.V. irradiated and mitomycin C treated strain B6 (Vervliet et al., 1975). It is possible that a mutation has also resulted in thermosensitivity in Ti plasmid conjugal transfer in this strain.

The influence of pH and phosphate on the two processes are also in contrast. Ti plasmid conjugal transfer occurred at all values of pH tested, from 5.6 - 7.7, with an optimum at pH 7.0, whereas *vir* gene induction has a much more restricted pH range, with an optimum at pH 5.7 (Rogowsky et al., 1987). Phosphate starvation is needed for efficient *virG* induction; an increase of phosphate concentration from 0.1 mM to 25 mM was found to cause about a 50 fold inhibition of *virG* transcriptional activity (Winans et al., 1988; Winans, 1990). But there was no significant difference detected in the transfer efficiency of pTi A6 when phosphate concentration was decreased from 100 to 5 mM.

Both acetosyringone (or related compounds) and glucose have been shown to be essential for *vir* gene induction (Stachel et al., 1985, Shimoda et al., 1990) but neither influenced Ti plasmid transfer.

It was noted that transposon mediated Tra$^-$ mutant M131, Tra$^-$Cfs$^-$ mutant M103, and Occ$^-$Tra$^-$Cfs$^-$ mutant M106 (Chapter 8) show normal pathogenicity on tomato plants when compared with their parent strain A6. In the case of nopaline strains, it is also reported that there is no appreciable difference in tumorigenesis between Tra mutants and their parent strain NT1(pTiC58Trac) or wild type strain C58 (von Bodman et al., 1989). Apparently, conjugation factor and other Tra functions are not required for T-DNA transfer.

The data presented in this thesis suggest that transfer of DNA to plant cells occurs by functions independent of those required for Ti plasmid conjugal transfer, although the likely importance of genes in the Vir region in CF biosynthesis might require this statement to be altered.

9.8 New Methods

As part of the research, some new methods have been established and all of them share common features of simplicity and efficiency.

A chemical method for synthesis of conjugation factor and its derivatives has been described in Chapter 4. The method involves only two steps that can be completed within one day; acyl Meldrum's acid intermediate is first prepared following the method of Oikawa et al. (1978), which is then reacted with homoserine lactone in the presence of pyridine to produce the *N*-acyl amide of homoserine lactone. The *lux* autoinducer of *V. fischeri* has also been chemically synthesized (Eberhard et al., 1981), but a few more days are required for completion of synthesis.

In Chapter 6, a rapid EtBr-phenol extraction procedure for purification of the Ti plasmid from *Agrobacterium* has been established to replace the time consuming CsCl-EtBr gradient ultracentrifugation method (Currier and Nester, 1976). The procedure exploits the differential solubility in phenol of supercoiled plasmid DNA campared with linear chromosomal DNA and RNA after EtBr treatment and can be used for small and large scale purifications. This simple and efficient procedure has greatly facilitated the molecular biological studies described in Chapters 7 and 8.

9.9 Conclusion

The research described in this thesis makes the following original contributions to our knowledge of Ti plasmid conjugal transfer in *Agrobacterium tumefaciens*:

(1) Discovery of a novel conjugation factor which is induced by the plant crown gall-produced conjugal opine, octopine and which behaves as a second messenger, transmitting the environmental signal to *tra* genes.

(2) Identification of the conjugation factor as *N*-ß-oxo-octanoyl-L-homoserine lactone, which is structurally very similar to the *lux* autoinducer, *N*-ß-oxo-hexanoyl-L-homoserine lactone, in a very dissimilar bacterium *Vibrio fischeri*,

suggesting that these *N*-acyl amides of homoserine lactone may be a group of widely conserved signal molecules for gene induction.

(3) Induction of *tra* gene activity by conjugation factor is highly specific; the lactone ring structure, the length and the nature of the acyl chain of the conjugation factor are very important for its biological activity.

(4) conjugation factor biosynthesis is encoded by two (or) more loci on the Ti plasmid. The *cfsA* locus has been mapped in a region close to *occ* genes.

(5) Demonstration by deletion analysis that conjugation factor biosynthesis is positively regulated by genes in the Occ region.

(6) Conjugation factor biosynthesis is also negatively regulated, the repressor being encoded by a locus located near the Occ region.

(7) Oxygen radicals are involved in the regulation of conjugation factor biosynthesis.

(8) Ti plasmid conjugal transfer can occur at temperatures above 30° C, indicating that Ti plasmid conjugal transfer does not share a thenmosensitive step with T-DNA transfer as previously proposed. It is also different from T-DNA transfer in that it occurs over a range of pH, from pH5.6 to pH7.7, with an optimum of pH7.0.

(9) Establishment of a simple and efficient method for synthesis of conjugation factor and its derivatives.

(10) A rapid and efficient method for the purification of Ti plasmids from *Agrobacterium* has been developed; it greatly facilitated the molecular biological studies on Ti plasmid conjugal transfer.

Besides revealing some very interesting issues in Ti plasmid conjugal transfer, such as whether the Vir region contains a *cfs* gene, how octopine and oxygen radicals regulate the production of conjugation factor, and how conjugation factor induces the expression of *tra* genes in *Agrobacterium*, the research described in this thesis has also led to some intriguing evolutionary questions:

(a) Conjugation factor is the first example of a second messenger in plasmid conjugal transfer system. Could other plasmid conjugation systems also have this kind of signal molecule?

(b). To what extent could these conjugation factor-type signals be conserved in different microorganisms? And what other functions are they possibly regulating?

(c). Most of the *cfs* genes and *tra* genes have been found in the Ti plasmid. Could the *lux* genes of *V. fischeri* also be plasmid-borne? If *V. fischeri* contains a plasmid, could the *lux* autoinducer also be a conjugation factor?

APPENDIX I. PUBLICATIONS

Zhang L, and Kerr A. 1991. A diffusible compound can enhance conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens*. J. Bacteriol. **173,** 1867-1872.

Zhang L, Murphy PJ, Kerr A, and Tate ME. 1993. *Agrobacterium* conjugation and gene regulation by N-acyl-L-homoserine lactones. Nature, London (In press)..

Zhang L, and Kerr A. 1993. Rapid purification of Ti plasmids from *Agrobacterium* by ethidium bromide treatment and phenol extraction. Letters in Applied Microbiology (In press).

Zhang L, Murphy PJ, Tate ME, and Kerr A. Oxygen radicals regulate conjugation factor biosynthesis in *Agrobacterium tumefaciens* (submitted).

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A Diffusible Compound Can Enhance Conjugal Transfer of the Ti Plasmid in *Agrobacterium tumefaciens*

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Several octopine strains of *Agrobacterium tumefaciens* were tested for Ti plasmid (pTi) transfer after induction by 400 μg of octopine per ml for 24 h. The strains could be divided into two groups, transfer efficient (Tra^{e}) and transfer inefficient (Tra^{ie}); the respective rates of transfer were 0.77×10^{-2} to 1.14×10^{-2} and 0.33×10^{-6} to 9.8×10^{-6} plasmid transconjugant per donor cell. Transfer efficiencies of Tra^{ie} strains were greatly increased when the time of induction was 72 h. A diffusible conjugation factor (CF) that can enhance conjugal transfer of pTi in *A. tumefaciens* was discovered when both Tra^{e} and Tra^{ie} donor strains were induced in the same plate. The evidence indicates that CF is a key factor affecting transfer efficiency of pTi but is not sufficient by itself to induce transfer. Tra^{c} mutants can produce CF constitutively, and Tra^{e} strains can produce it after induction by low octopine concentrations. The transfer efficiency of Tra^{ie} strains was greatly increased by adding CF to the induction medium. The thermosensitive strain B6S3, which normally cannot conjugate at temperatures above 30°C, could transfer pTi efficiently at 32 and 34°C in the presence of CF. Production of CF is dependent on the presence of pTi but appears to be common for different opine strains; it was first detected in octopine strains, but nopaline strains also produced the same or a similar compound. CF is very biologically active, affecting donor but not recipient bacterial cells, but CF does not promote aggregation. Data suggest that CF might be an activator or derepressor in the conjugation system of *A. tumefaciens*. CF is a dialyzable small molecule and is resistant to DNase, RNase, protease, and heating to 100°C for 10 min, but autoclaving (121°C for 15 min) and alkaline treatment removed all activity.

There are two DNA transfer processes associated with the Ti plasmid (pTi) of *Agrobacterium tumefaciens*; one is T-DNA transfer from *A. tumefaciens* to a host plant cell (34, 35), and the other is pTi transfer from *Agrobacterium* donor cells to recipient cells (16, 21, 27). Evidence indicates that the basic processes of these two DNA transfers are similar; both involve activation of gene expression in response to external stimuli (21, 22) and, presumably, conjugal transfer of single-stranded DNA from a donor cell to a recipient cell (reviewed in references 24, 33, and 35). Interestingly, these two DNA transfer processes are closely related by their own unique functions; DNA is transferred from bacterial cell to plant cell, and the latter is directed to grow and divide rapidly and to produce opines (reviewed in references 4, 34, and 35), which play a crucial role in the whole process. Most opines are imino acids. Not only do they provide a more or less reserved food supply for the inciting bacteria, but they also induce bacterial conjugation and pTi transfer (11, 15, 27). This was the first example of substrate-induced conjugation in bacteria. Many different opines are synthesized by different strains of *A. tumefaciens*, and conjugation induction is relatively specific. Octopine induces conjugation by octopine strains, but agropine, which is also synthesized in crown gall tissue induced by these strains, does not. Octopine is also synthesized in grapevine crown galls incited by many biovar 3 strains of *A. tumefaciens* (26), but it does not induce their conjugation. Nopaline strains are not induced to conjugate by octopine or even by nopaline but by another opine, agrocinopine A, which is also present in crown gall tissue induced by these strains (7, 27).

Although opine induction of conjugal transfer in *A. tumefaciens* has been known for many years, we still know very little about the process. Early work was mainly on substrate induction of pTi conjugal transfer (reviewed in reference 14). It was found that constitutive opine catabolic (Occ^{c}) mutants are also transfer constitutive (Tra^{c}) (9, 17, 27). This suggests a model in which a repressor blocks common operator sites in the promoter region of the two loci and in which the opine derepresses the operon by binding to the repressor (14, 27). However, Klapwijk et al. (16, 17) presented evidence that a single insertion mutant could produce the phenotype Occ^{-} Tra^{-}. Therefore, a positive control gene might also be involved in the regulation of pTi conjugal transfer. More recent work with a nopaline strain identified *tra* genes in three separate loci on pTi (3). The combined size of the three loci was only about 9 kb, which seems rather small when compared with about 40 kb in the *vir* region required for T-DNA transfer (23) and with the *tra* region of the *Escherichia coli* F plasmid, which is more than 33 kb (33). Clearly, more work is needed to reveal the mechanism of *Agrobacterium* pTi conjugal transfer.

The conjugative mechanism of pTi transfer is of interest, not only because opine-induced pTi transfer is important in crown gall disease epidemics, but also because characterization of pTi transfer would help to elucidate whether there is any relationship between it and T-DNA transfer, a suggestion made on several occasions (3, 10, 25, 29, 31). In this paper we describe (i) the discovery of a diffusible conjugation factor (CF) that is important in the regulation of pTi conjugal transfer, (ii) factors influencing the synthesis of CF, (iii) some of the chemical and physical characteristics of CF, and (iv) the way in which CF can influence conjugation.

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TABLE 1. Bacterial strains and plasmids

Strain	Plasmid	Description[a]
A6	pTiA6	Octopine catabolic, supplied by G. Morel
NCPPB1001	pTiNCPPB1001	Octopine catabolic, supplied by NCPPB
K608	pTiB6::Tn5	Octopine catabolic, T-DNA::Tn5, Tra^{ci} Chl^{r}, C58 pTi^{-} background, supplied by J. G. Ellis
K794	pTiB6::Tn5	Octopine catabolic, TR-DNA::Tn5, Tra^{c} Rif^{r} Str^{r}, C58 pTi^{-} background, supplied by J. G. Ellis
K804	pTiB6::Tn5	Octopine catabolic, TR-DNA::Tn5, Tra^{c} Rif^{r} Str^{r}, C58 pTi^{-} background, supplied by J. G. Ellis
B6	pTiB6	Octopine catabolic, supplied by G. Morel
Ach5	pTiAch5	Octopine catabolic, supplied by J. Schell
B6S3	pTiB6	Octopine catabolic, a phage-sensitive isolate from UV-irradiated and mitomycin C-treated B6 (16), supplied by J. Tempé
T37	pTiT37	Nopaline catabolic, Tra^{ci}, supplied by G. Morel
K323	pTiT37	Nopaline catabolic, Tra^{c} colony of strain T37 isolated from octopine medium, our laboratory
K749		C58 pTi^{-} pAt^{-} Rif^{r} Str^{r}, supplied by A. Kondorosi
K749(pTiK608)	pTiB6::Tn5	Octopine catabolic, transconjugant from K608 × K749, this study
K608C1		pTiB6::Tn5 was cured from strain K608, this study

[a] Abbreviations: Chl^{r}, chloromycetin resistant; Rif^{r}, rifampin resistant; Str^{r}, streptomycin resistant; NCPPB, National Collection of Plant Pathogenic Bacteria, United Kingdom.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. Strain K749, a plasmidless derivative of strain C58, was used as the recipient strain in all experiments. Strains were maintained on YM medium (8) and, if necessary, supplemented with antibiotics.

The minimal salts medium of Petit and Tempé (20) was the basis of induction and selective media; 200 μg of octopine per ml and 1 mg of glucose per ml were added except where otherwise stated, and 1 mg of $(NH_4)_2SO_4$ per ml was used in induction medium to replace octopine in control experiments. The selective medium was supplemented with 50 μg of rifampin and 500 μg of streptomycin per ml. The induction and selective media were adjusted to pH 7.0.

Chemicals and enzymes. Opines used in this study were synthesized by M. E. Tate and J. Tempé. L-Proline, D-proline, L-arginine, L-ornithine, protease type XIV, RNase type 1-A, and DNase type III are the products of Sigma. The enzymes were dissolved in sterile distilled water before use.

Conjugation. (i) Drop mating. On day 1, fresh cultures of donor and recipient strains grown on YM plates for 1 or 2 days were suspended in 5 ml of sterile distilled water and adjusted to an optical density at 600 nm of 0.4, and 200 μl of recipient suspension was spread on to selective plates; 30 μl of each donor strain was inoculated into a culture tube that contained 1 ml of liquid induction medium. Donors were incubated with shaking at 25°C. The induction time was 24 h except when the effect of induction time on conjugation was tested. Recipient plates were kept at 28°C for 24 h. On day 2, donor strains were suspended, and a 10-fold dilution series was prepared; 10-μl samples of 10^{0} to 10^{-5} dilutions were spotted in triplicate onto a lawn of the recipient growing on selective medium; 10 μl of 10^{0} dilution of donor cells was spotted on selective medium as a control. To determine the number of viable donor cells, 10-μl samples of 10^{-5}, 10^{-6}, and 10^{-7} dilutions were spotted onto YM plates. Plates were incubated at 28°C, and then the numbers of donors and transconjugants were recorded after 2 and 5 days, respectively. Since the recipient strain cannot utilize octopine, transconjugants are easily distinguished. Transfer efficiencies of donor strains were calculated as the number of transconjugants observed per donor cell applied.

(ii) Replica plating mating. On day 1, a recipient strain was spread on selective medium as above, and donors were applied as patches to solid induction medium. Both recipient and donors were incubated at 28°C for 24 h. On day 2, induced donors were replica plated onto the recipient lawn. Transconjugants were observed after incubation at 28°C for 4 or 5 days.

Octopine utilization. Test strains were inoculated into YEB (30) and incubated for 24 h at 25°C. The optical density at 600 nm of these fresh inocula was adjusted to 0.4, and 0.5 ml of each inoculum was added to 3 ml of liquid induction medium containing 50 μg of octopine per ml. The presence of octopine in the medium was determined by the colorimetric method of Lippincott et al. (18) at 0 and 30 min and at 1, 2, 5, 10, and 24 h after inoculation.

CF preparation and CF activity bioassay. CF filtrate was prepared as follows: strain K608 was inoculated into 10 ml of liquid induction medium containing 400 μg octopine per ml and incubated at 25°C for 24 h with shaking. The culture was centrifuged, and the supernatant was collected and sterilized by membrane filtration (0.2-μm pore size; Millipore). The presence of octopine was determined. When CF induction by other opines and chemicals was tested, the opines and chemicals also were used at 400 μg/ml, and the uninoculated media were used as controls to make sure that enhanced pTi transfer efficiency was due to CF rather than to the added opines and chemicals.

The same procedure was used to examine the CF-producing ability of all Tra^{c} and partially transfer constitutive (Tra^{ci}) strains used. For the inducible strains A6, NCPPB 1001, B6, Ach5, K749, and plasmidless strain K608C1, which fail to produce CF in liquid medium, the replica plate mating method was used to determine CF production; the CF-producing strain and CF activity indicator strain (B6S3) were patched side by side at a distance of 1.2 cm (Fig. 1).

Strain B6S3 was selected to indicate CF activity because it cannot transfer pTi when the concentration of octopine in the induction medium is 200 μg/ml (Table 2). Drop mating and replica plate mating methods, respectively, were used in quantitative and qualitative assays of the promoting effect of CF on pTi conjugal transfer. In the qualitative assay, 400 μl of a CF preparation was added to an agar induction plate containing 200 μg of octopine per ml; after drying, strain B6S3 was patched onto the plate, incubated for 24 h at 28°C, and replica plated onto a lawn of the recipient on selective medium. In the quantitative assay, 200 μl of CF filtrate and 30 μl of a cell suspension of strain B6S3 were added to 1 ml of liquid induction medium (200 μg of octopine per ml),

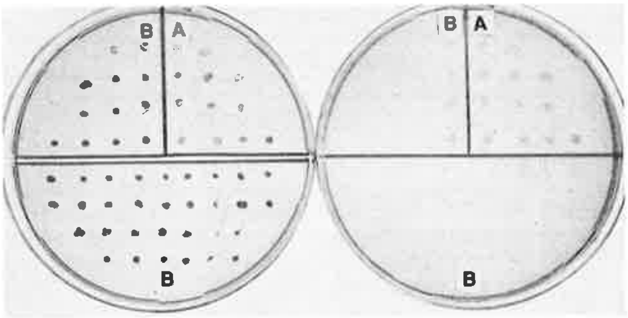

FIG. 1. A diffusible conjugation factor produced by strain K608 (A) promotes pTi conjugal transfer of strain B6S3 (B). Left, Induction plate; right, selective plate showing transconjugants. Double lines indicate the location of the ditch.

incubated for 24 h, and diluted; 10-μl drops were added to a lawn of the recipient on selective medium.

Aggregation assay. The method of Dunny et al. (6) was followed to determine whether CF could induce aggregation between donor cells, recipient cells, or combined donor and recipient cells; 1 ml of CF preparation plus 800 μl of fresh liquid induction medium plus 200 μl of late-log-phase cultures of donor cells, recipient cells, or a 1:1 mixture of donor and recipient were mixed, incubated at 28°C, and monitored for aggregation.

RESULTS

Sensitivity of strains to octopine induction. Octopine concentrations from 200 to 1,400 μg/ml were tested on several strains for induction of pTi conjugal transfer. The sensitivity of strains to octopine induction of conjugation was variable (Table 2). Transfer-efficient (Tra^{e}) strains were very sensitive; octopine at a concentration of 200 μg/per ml induced strain A6 to transfer pTi at a rate of 0.52×10^{-2} per donor cell. Other strains were transfer inefficient (Tra^{ie}) and much less sensitive; the transfer efficiency of strain B6S3 was only 0.39×10^{-4} transconjugant per donor at an octopine concentration of 1,400 μg/ml. Rates of octopine utilization by strains K608 and B6S3 were measured, but no significant difference was detected (data not shown).

Influence of induction time. The influence of time of induction by octopine on pTi conjugal transfer was pronounced. For the Tra^{ie} strains B6, Ach5, and B6S3, induction for 24 h by 200 μg of octopine per ml did not result in any pTi transfer (Table 3), but when the induction time was increased to 72 h, the transfer efficiencies increased markedly.

TABLE 3. Effect of time of induction by 200 μg of octopine per ml on pTi conjugal transfer

Strain	Period of induction (h)	Transfer efficiency (transconjugants per donor)
B6	24	$<1 \times 10^{-7}$
	72[a]	0.19×10^{-3}
Ach5	24	$<1 \times 10^{-7}$
	72[a]	0.35×10^{-3}
B6S3	24	$<1 \times 10^{-7}$
	72[a]	0.57×10^{-5}
A6	24	0.50×10^{-2}

[a] The transfer efficiencies in control experiments were $<1 \times 10^{-7}$ transconjugant per donor.

Tra^{e} strains are fully competent after 24 h induction by octopine. Shorter periods were tested with strain A6; no pTi transfer occurred after 5 h of induction ($<1 \times 10^{-7}$ transconjugant per donor). However, when after 5 h of induction, bacteria were grown on a noninducing substrate for another 19 h before plating on selective medium, pTi transfer occurred (0.59×10^{-6} transconjugant per donor). The corresponding rates following 10 h of induction followed by 0 or 14 h of growth on noninducing substrate were 0.16×10^{-6} and 0.23×10^{-4} transconjugant per donor. This compared with a rate of 0.83×10^{-2} transconjugant per donor after induction for 24 h.

A new CF. In our preliminary experiments to investigate the effect of temperature on pTi transfer by different strains, it was noted that, under some conditions, transconjugant colonies of Tra^{ie} strains appeared only when adjacent to Tra^{e} transconjugants. It appeared that Tra^{e} strains were producing a diffusible compound that could stimulate pTi transfer. This was confirmed by a "ditch plate" experiment in which a Tra^{ie} strain was separated from a Tra^{e} strain on an agar induction plate by removing a thin slice of agar. Figure 1 shows the effect of chemical diffusion from strain K608 to strain B6S3. When the agar slice was not removed, transconjugant colonies of strain B6S3 appeared only when adjacent to strain K608. When the two strains were separated by a ditch in the induction medium, no transconjugants of strain B6S3 could be seen on the selective medium. The existence of this diffusible conjugation factor was confirmed by chromatography and high-voltage paper electrophoresis (data not shown).

Influence of CF on pTi transfer efficiency. Several octopine strains were tested for synthesis of CF after induction by various chemicals for various periods. There was a strong correlation between CF production and pTi conjugal transfer efficiency (Table 4). Tra^{e} strains A6 and NCPPB1001 produced CF after being induced for 24 h by 200 μg octopine per ml on agar plates, but Tra^{ie} strains Ach5 and B6 could only

TABLE 2. Transfer efficiency of octopine strains

Strain	Transfer efficiency (no. of transconjugants per donor) at the following concn of octopine (μg/ml):					Classification based on sensitivity
	0	200	400	1,000	1,400	
A6	$<1 \times 10^{-7}$	0.50×10^{-2}	0.77×10^{-2}	0.83×10^{-2}	0.97×10^{-2}	Tra^{e}
NCPPB1001	$<1 \times 10^{-7}$	NT[a]	1.14×10^{-2}	NT	NT	Tra^{e}
K608	0.97×10^{-6}	NT	0.79×10^{-2}	NT	NT	Tra^{e}
B6S3	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	0.33×10^{-6}	0.28×10^{-5}	0.39×10^{-4}	Tra^{ie}
Ach5	NT	$<1 \times 10^{-7}$	0.98×10^{-5}	NT	0.27×10^{-3}	Tra^{ie}
B6	NT	$<1 \times 10^{-7}$	0.57×10^{-5}	NT	0.14×10^{-3}	Tra^{ie}

[a] NT, Not tested.

TABLE 4. CF production by different octopine strains of *A. tumefaciens*, with and without induction

Strain	Transfer system	Inducing chemical (per liter)	Induction time (h)	CF activity[a]
K794	Tra^c	$(NH_4)_2SO_4$ (1 g)	24	+
K804	Tra^c	$(NH_4)_2SO_4$ (1 g)	24	+
K608	Tra^{ci}	$(NH_4)_2SO_4$ (1 g)	24	−
		Octopine (200 mg)	24	+
A6	Tra^e	Octopine (200 mg)	24	+
NCPPB1001	Tra^e	Octopine (200 mg)	24	+
B6	Tra^{ie}	Octopine (400 mg)	24	−
		Octopine (400 mg)	72	+
Ach5	Tra^{ie}	Octopine (400 mg)	24	−
		Octopine (400 mg)	72	+

[a] CF production was determined by using a CF filtrate preparation for strains K794 and K804 and by the ditch plate method for the other strains.

TABLE 5. pTi is required for CF production

Strain	pTi	Inducing chemical[a] (400 μg/ml)	CF production
K749	−	Octopine	−
	−	Nopaline	−
K749(pTiK608)	+	Octopine	+
	+	Nopaline	+
K608C1	−	Octopine	−

[a] Induction time is 24 h.

synthesize CF after being induced for 72 h. Tra^c strains could produce CF constitutively, but in the Tra^{ci} strain K608 CF production was detected only after induction by octopine.

Strain B6S3 was induced by a wide range of octopine concentrations with and without the addition of CF. The presence of CF had a marked influence on conjugation over the whole range of octopine concentrations (Fig. 2). However, when no octopine was present, CF did not induce conjugation.

Several strains were tested for production of CF in both liquid and solid induction media. All Tra^c and Tra^{ci} mutants tested (K608, K794, K804, T37, and K323) produced CF in both solid and liquid media, but with the wild-type inducible strains A6, NCPPB1001, B6, and Ach5, CF production was detected only in solid medium.

Relationship of CF to nopaline strains. pTi can be divided into octopine, nopaline, and agropine types based upon the opines they produce and catabolize (14, 28). Although different opine strains need specific opines for induction of pTi conjugal transfer (7, 14, 28), strong homology between Tra regions of these strains has been observed by several groups (5, 12, 13). This indicates that there are some *tra* genes conserved in different opine-type Ti plasmids. When the nopaline Tra^c and Tra^{ci} strains K323 and T37 were grown for 24 h in liquid induction medium with 1 g of $(NH_4)_2SO_4$ per liter, a substance was produced that promoted conjugal transfer in the octopine strain B6S3.

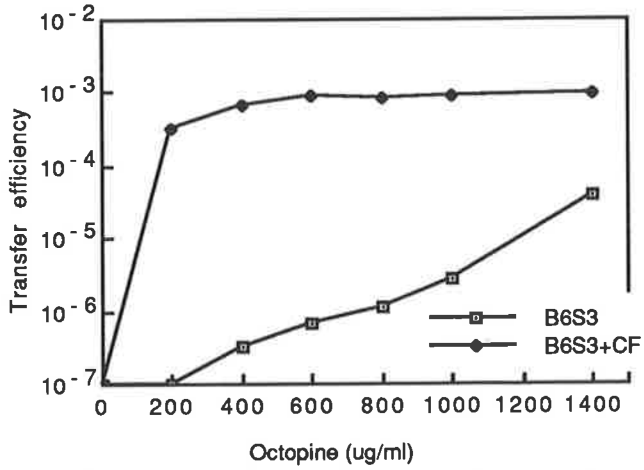

FIG. 2. CF promoting pTi transfer efficiency by strain B6S3.

Requirement of pTi for CF production. pTi is required for the production of CF. When pTi was cured from strain K608, it lost CF production ability; conversely, strain K749 gained this ability along with pTi from strain K608 (Table 5).

Imino acids induce CF production in strain K608. Because nopaline strains can produce a compound that promotes pTi transfer by an octopine strain, it appeared possible that CF induction also was not highly specific. A wide range of chemicals was tested by using the Tra^{ci} strain K608 as a producer. All of the imino acids tested could induce strain K608 to produce CF, although some of them could not be utilized by strain K608 or were utilized only poorly (Table 6). Octopine was the most efficient inducer, followed by nopaline and noroctopinic acid. L- and D-proline, which are not opines, could also efficiently induce production of CF. But L-arginine and pyruvate, the components of octopine (19), had no inducing effect.

Effect of temperature on CF production by strain B6S3. Tempé et al. (29) found that strain B6S3 could not transfer its pTi at a temperature above 30°C. The data in Table 7 confirm their observation. When the induction temperature was higher than 30°C, transfer efficiency of strain B6S3 was less than 1×10^{-7} transconjugant per donor. This was not due to a difference in octopine catabolism at the various temperatures (data not shown). However, when CF was added to the induction medium, strain B6S3 could transfer pTi at 32 and 34°C at rates of 0.93×10^{-3} and 0.55×10^{-4} transconjugant per donor, respectively.

Properties of the diffusible CF. Aliquots (1 ml) of CF

TABLE 6. CF production by strain K608 after induction by various imino acids

Chemical	Catabolism by strain K608	CF activity[a]
Octopine	+	0.83×10^{-3}
Norooctopinic acid[b]	Very poor	0.87×10^{-4}
Homooctopine[b]	−	0.61×10^{-5}
Agropine	+	0.35×10^{-4}
Deoxymannityl glutamic acid[b]	Poor	0.42×10^{-5}
Nopaline[b]	−	0.97×10^{-4}
Allo-nopaline[b]	−	0.36×10^{-5}
L-Proline	+	0.72×10^{-4}
D-Proline	+	0.54×10^{-4}
L-Arginine and pyruvate	+	$<1 \times 10^{-7}$
Control[c]	+	$<1 \times 10^{-7}$

[a] CF activity is expressed as pTi transfer efficiency (transconjugants per donor) by strain B6S3.

[b] When the inducing chemical could not be catabolized by strain K608, the medium was supplemented with 1 mg of $(NH_4)_2SO_4$ per ml to provide a source of nitrogen.

[c] Control treatment consisted of 1 mg of $(NH_4)_2SO_4$ per ml.

TABLE 7. pTi conjugal transfer by strain B6S3 at a range of temperatures, with and without CF

Induction treatment	Transfer efficiency (transconjugants per donor) at the following temp (°C):					
	24	28	30	32	34	37
Octopine (2.4 mg/ml)	1.01×10^{-3}	0.94×10^{-3}	0.37×10^{-3}	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$
Octopine (2.4 mg/ml) + CF	1.04×10^{-3}	1.12×10^{-3}	0.88×10^{-3}	0.93×10^{-3}	0.55×10^{-4}	$<1 \times 10^{-7}$

preparation were treated (i) with 2 N NH_4OH at 45°C for 2 h, (ii) by autoclaving at 121°C for 15 min, (iii) with DNase (500 μg/ml), (iv) with RNase (500 μg/ml), or (v) with protease (50 μg/ml). All enzymatic treatments were at 37°C for 20 min followed by 100°C for 10 min. After treatments, 400 μl from each treatment was used to assay CF activity. Only treatments (i) and (ii) removed activity. CF is a small molecule; the filtrate obtained after passing CF through Centricon 10 had lost no activity. CF is also dialyzable; when we dialyzed a CF preparation overnight against liquid induction medium lacking octopine, CF activity was lost from the solution within the membrane but could be detected in the external liquid after concentration.

Mode of action. Dunny et al. (6) reported that recipient strains of *Streptococcus faecalis* produce a diffusible clumping-inducing agent that causes strains carrying certain conjugative plasmids to aggregate. The fact that CF can diffuse from bacterial donor cells prompted us to consider that CF might have some effect on recipient cells. Therefore, 400 μl of CF preparation was added to the selective medium before the recipient was added; the culture was incubated for 24 h at 28°C and then drop mated with strain B6S3 that had been induced for 24 h by 200 μg of octopine per ml. However, the result showed no difference in transfer efficiency between that treatment and the negative control. CF did not induce any measurable aggregation between donor cells, recipient cells, or a mixture of donor and recipient cells.

Although quantitative measurement of CF concentration is impossible at this stage, there can be little doubt that CF is a highly active molecule. A 50-fold dilution of CF preparation can still enhance pTi transfer efficiency in Tra^{ie} strain B6S3 by more than 1,000 times when coinduced by 200 μg of octopine per ml.

DISCUSSION

Our results show that octopine strains of *A. tumefaciens* can be placed in two distinct groups, based on their conjugal transfer efficiency. The distinction is also apparent in their response to different concentrations of octopine and in the time required for induction. However, the distinction appears to be eliminated, or at least reduced, when inefficient strains are exposed to a diffusible CF produced by the efficient strains. The discovery of CF explains the difference in pTi transfer efficiency of Tra^{e} and Tra^{ie} strains and might also help to elucidate the mechanism of opine-induced pTi conjugal transfer.

Tempé and Petit (27) proposed a model to explain regulation of genes controlling opine catabolism and Ti plasmid transfer. In their model, opines induce conjugal transfer by binding to the repressor of *tra* genes. The fact that CF can significantly enhance pTi transfer efficiency in the presence of a low concentration of octopine but is not able by itself to induce transfer indicates that the regulation of pTi transfer is more complicated than that model. Presumably CF is an activator or derepressor of the system, but at present the mode of action is unclear. Its production clearly involves pTi. When the CF-producing strain K608 was cured of its pTi, it lost CF production ability; conversely, strain K749, which is a plasmidless strain, gained this ability after it received the pTi from strain K608.

A temperature greater than 30°C has been reported to inhibit both *vir* gene induction (1, 2) and pTi transfer (29), and therefore it was suggested that T-DNA transfer and pTi conjugal transfer might share a common thermosensitive step. But our results show that temperatures from 24 to 34°C have no significant effect on conjugal transfer by all strains we tested (data not shown) except strain B6S3. Strain B6S3 is a thermosensitive strain; when induction temperature is above 30°C, no pTi transfer is detected. This agrees with the observation of Tempé et al. (29). However, when CF was added to the induction medium, the pTi transfer efficiencies of strain B6S3 were 0.93×10^{-3} and 0.55×10^{-4} transconjugant per donor at 32 and 34°C, respectively. These data suggest that there is a thermosensitive step involved in CF production by strain B6S3. Strain B6S3 is a phage-sensitive isolate from UV-irradiated and mitomycin C-treated strain B6 (32). It is likely that a mutation has also resulted in thermosensitivity in conjugal transfer in this strain.

CF is probably a common factor among different opine strains. Besides octopine strains, nopaline Tra^{c} and Tra^{ci} strains K323 and T37 were able to produce CF or at least a very similar compound. Other evidence supports this hypothesis; all opines tested were able to induce strain K608 to produce CF, although their effect varied. Imino acids L-proline and D-proline could also induce CF production. But the amino acid L-arginine, a substituent of octopine (19), had no effect on CF induction. Results suggest that the imino group might be the basic structure needed for CF induction in strain K608. But at this stage, we cannot determine whether imino acids other than octopine can also induce CF production in wild type-inducible strains, because these strains do not exude CF into liquid induction medium under the culture conditions we used.

CF is a low-molecular-weight compound, as indicated by its dialyzable property and because its activity is not affected by passage through a Centricon 10 membrane, which is a microconcentrator designed to retain molecules with molecular weights higher than 10,000. CF was resistant to nuclease and protease, but activity was destroyed by autoclaving and by alkaline treatment. So it is clearly not a nucleic acid or protein; at present, its nature is not clear.

ACKNOWLEDGMENT

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NATURE — Ref 9915/1 — Kerr — BIO

Agrobacterium conjugation and gene regulation by *N*-acyl-L-homoserine lactones

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CONJUGAL opines secreted by crown gall tumours induce strains of *Agrobacterium tumefaciens* that are donors of Ti plasmids to produce a diffusible conjugation factor[1]. This enhances the conjugal transfer efficiency of the Ti plasmid in other strains of *A. tumefaciens*. This factor behaves as a secondary messenger, transmitting the environmental information to *tra* genes. Here we report the use of spectrometry to show that ~~one of these~~ factor~~s~~ this is identical to synthetic *N*-(β-oxo-octan-1-oyl)-L-homoserine lactone and confirm that the synthetic compound is biologically active. *N*-(Hexan-1-oyl)-L-homoserine lactone has also been detected. A closely related molecule, *N*-(β-oxo-hexan-1-oyl)-L-homoserine lactone, autoinduces bioluminescence in the distantly related bacterium, *Vibrio fischeri*[2]. *N*-acyl-homoserine lactones thus seem to be conserved and in which the length and nature of the lipophilic acyl chain determines the biological function to be regulated. Mutants that do not produce the factor fail to conjugate unless supplied with it in the induction medium (our unpublished data). These data indicate that the conjugation factor is an autoinducer and a key signal molecule in the conjugation system of *A. tumefaciens*. It is, to our knowledge, the first example of a second messenger molecule in a bacterial conjugation system.

Conjugation factor (CF) is present in the culture supernatent of transfer constitutive strains of *A. tumefaciens*[1]. Its purification using high-performance liquid chromatography (HPLC) yielded two active components, CF1 and CF2. Examination of these fractions by chemical ionization quadrupole mass spectrometry showed strong quasimolecular (M+H) ions, with an m/z of 242 for CF1, and for the less homogeneous CF2 fraction, ions at m/z 274, 214 and 200. All of these even M+H ions were consistent with the presence of nitrogenous components. For CF1 this was confirmed by infrared spectrometry, which showed amide I and II carbonyl absorptions at 1,646 and 1,540 cm^{-1} as well as characteristic absorptions near 1,780 cm^{-1} and 1,714 cm^{-1} assignable to the presence of γ lactone and ketonic carbonyls[2]. Acid hydrolysis of CF1 yielded an amino acid which comigrated with homoserine when separated by high-voltage paper electrophoresis. Daughter ion tandem mass spectrometry of the M+H ions at 242 m/z in CF1, and M+H ions at 274, 214 and 200 in CF2 fraction showed related fragmentations after helium collisional activation. Except for the 214 parent ion, which showed a daugher ion at 113 instead of 99 as well as ions at 102 and 141, each daughter ion set from the parent ions at 242, 274 and 200 m/z, displayed prominent 99, 102 and 141 m/z ions. Comparison of the corresponding electron impact data with those reported[2] suggested a closely related set of homoserine lactone derivatives. These data taken together with the reported[3] homology of TraR in *A. tumefaciens* with LuxR in *Vibrio fischeri*, which requires *N*-(β-oxo-hexan-1-oyl)-L-homoserine lactone for gene induction, indicate that a common homoserine lactone moiety is associated with gene regulation in these distantly related species.

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The known luciferase autoinducer β-oxo-hexanol homoserine of *V. fischeri*[2] (Fig. 1, structure $n = 0$) was synthesized to compare its mass spectrum with that of natural CF. The comparison confirmed its relatedness, but nonidentity with the tandem ion mass spectrometry daughter ion spectra from the 214 m/z parent ion in the CF2 fraction. But data from the homogeneous CF1 fraction were consistent with a -$(CH_2)_2$- extension of the lipophilic side chain of the luciferase autoinducer, β-oxo-octanoyl homoserine lactone (Fig. 1, $n = 1$). This was also synthesized. These molecules have very similar infrared spectra with the four characteristic carbonyl signals[2]. In the case of CF1, sufficient homogeneous material was obyained to show that all the measured properties (ultraviolet, infrared, ^{1}H, nuclear magnetic resonance (NMR), ^{13}C NMR; chromatographic parameters data not shown) and biological activity (Fig. 2) were virtually indistinguishable from those of the synthetic sample. The tandem mass spectrometric framents of the M+H ion of m/z 200 present in the CF2 fraction suggested it was the fully reduced hexanoyl amide of homoserine lactone and the daughter ion fragmentation patterns were indistinguishable from those of a synthetic sample of the hexanoyl amide.

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Figure 2 shows *tra* gene activity induced by the natural CF1 from *A. tumefaciens* and by synthetic *N*-β-oxo-octanoyl-, *N*-β-oxo-hexanoyl-, *N*-β-oxo-decanoyl-, *N*-octanoyl- and *N*-hexanoyl amides of L-homoserine lactone. Only the synthetic *N*-β-oxo-octanoyl-L-homoserine lactone shows identity in gene induction activity with the natural product and accounts fully for the observed activity of fraction CF1. Although masses corresponding to the *N*-hexanoyl amide were detected by mass spectrometry in the biologically active CF2 fraction from the HPLC fractionation, the synthetic product had insufficient activity on a molar basis to account for the (autoinducing) activity of this fraction. The close relationship of the daughter ions from the 274 parent ion to those of synthetic conjugation factor m/z 242 and in particular the presence of a strong fragment ion at 134 instead of the ion corresponding to a protonated homoserine lactone at 102, suggested that this fraction contained the methyl ester of the conjugation factor derived as a methanolic-solvent transesterification artefact. This was confirmed by the chromatographic behaviour, CI daughter ion mass spectra, infrared spectra and biological activity of the synthetic acyclic methyl ester of CF1.

The remarkable feature of the data in Fig. 2 is that extension (Fig. 1, $n = 2$) or diminution (Fig. 1, $n = 0$) of the chain length or conversion of the beta keto groups (Fig. 1, $n = 0$ and $n = 1$) to methylene groups is sufficient to reduce the inducing activity by four orders of magnitude or more compared to that of the β-oxo-octanoyl derivative ($n = 1$). A similar specificity was reported for analogues of the autoinducer of bioluminescence in *V. fischeri*[4].

The facts that *N*-β-oxo-hexanoyl-homoserine lactone is the autoinducer for luminescence activity in *V. fischeri*[2] and for antibiotic biosynthesis in *Erwinia*[5], that *N*-β-hydroxy-butanoyl-L-homoserine lactone is the autoinducer for luminescence in *V. harveyi*[6] and that *N*-β-oxo-octanoyl-L-homoserine lactone is the autoinducer for conjugation in *A. tumefaciens*, suggest that *N*-acyl homoserine lactones may be a widely conserved signal for gene regulation. ☐

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FIG. 1 Structural formulae for *N*-β-oxo-acyl-amides of L-homoserine lactone, $n=0$, 1 and 2 correspond to the *N*-β-oxo-hexanoyl, octanoyl and decanoyl amides. For isolation of the conjugation factor CF1 ($n=1$), strain K794, a transfer constitutive octopine strain of *A tumefaciens*, was grown in Petit's liquid minimal medium[7] for 2 days at 28 °C, using proline and mannitol as the nitrogen and carbon sources. Presence of CF activity was determined by induction of a *Tra::lacZ749* reporter gene fusion in strain NT-1 (supplied by S. Farrand[3]). CF was extracted from K794 bacterial culture filtrates by ethyl acetate. Partial purification of the concentrated extracts was achieved by flash chromatography on silica gel using ethyl acetate as eluant[8], followed by C18 reversed-phase open column flash chromatography eluted isocratically with 30% (v/v) methanol/water. Evaporation of the active fractions gave a yellowish white solid (3 mg L^{-1} culture). Further purification by C18 reversed-phase HPLC (analytical 25 cm, 4 mm inner diameter) eluted (1 mL min^{-1}) with a linear gradient of 30–45% methanol, gave two separate biologically active components, labelled CF1 (retention time 13.38 min) and CF2 (retention time 11.4 min). A quantitative β-galactosidase assay[9], was used to measure CF activity. Synthetic CF1 ($n=1$) was obtained in 49% yield in a one-step procedure using L-homoserine lactone HCl (1 mmol) in pyridine (10 ml) at 130 °C for 45 min with the acyl Meldrum's acid intermediate of Oikawa[10] (1.5 mmol). Cognate preparations proceeded smoothly with the appropriate intermediate[10]. Saturated acyl amides were prepared from the L-homoserine lactone HCl and corresponding acyl chloride in pyridine under the same conditions.

FIG. 2 Selective induction of *tra* gene activity by *N*-acyl-amides of L-homoserine lactone. Natural: CF1 (●); synthetic: *N*-β-oxo-octanoyl- (○), *N*-β-oxo-hexanoyl- (□), *n*-β-oxo-decanoyl- (■), *N*-octanoyl- (△) and *N*-hexanoyl-amides (▲) of L-homoserine lactone. β-Galactosidase activity was expressed as Miller units[9]. *N*-acyl-amides of L-homoserine lactone were added to the minimal medium[7] plus $(NH_4)_2SO_4$ (0.2, w/v) and mannitol (0.2%, w/v) and then inoculated with a 1:40 dilution of a late log phase culture (absorbance at 620 nm ≅ 0.6) of NT-1 (*traR, tra::lacZ749*)[3]. After 22 h of incubation at 25 °C, cells were collected by centrifugation and assayed for β-galactosidase activity[11].

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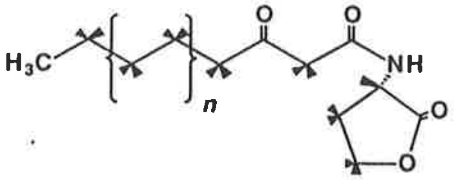
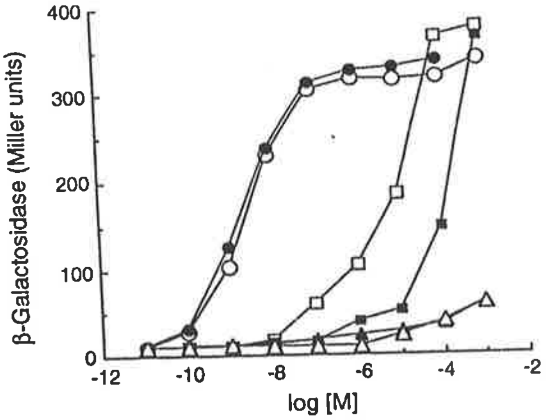


NATURE 9915 KERR-1 62%



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Letters in Applied Microbiology 1993, **16**, 000–000

Rapid purification of Ti plasmids from *Agrobacterium* by ethidium bromide treatment and phenol extraction

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An efficient method is described for the purification of Ti plasmid DNA from *Agrobacterium*. The procedure is based on the relative binding capacity of ethidium bromide to supercoiled plasmid DNA and linear DNA and on the high solubility of ethidium bromide in phenol. Following treatment with ethidium bromide, more than 87% of linear chromosomal DNA and most of the RNA was present in the phenol phase, while 91% of Ti plasmid DNA was recovered from the aqueous phase. The Ti plasmid DNA was sufficiently pure for restriction endonuclease analysis and cloning. The procedure is simple, fast and provides eight times higher yield than the standard isopycnic ultracentrifugation method.

The Ti plasmid of *Agrobacterium tumefaciens* encodes many genes required for different biological functions such as crown gall induction, conjugal transfer and opine catabolism (Kerr 1992). To facilitate molecular studies on the biological functions encoded by the Ti plasmid, an efficient method is needed to purify plasmid DNA from *Agrobacterium*. Various rapid procedures to prepare relatively pure plasmid DNA from small volumes of *Escherichia coli* cultures have been developed for restriction mapping purposes (He *et al.* 1989; Holmes and Quigley 1981; Saunders and Burke 1990) but for purification of Ti plasmids from *Agrobacterium*, these procedures often result in low yield or an impure product. This is possibly due to the fact that the Ti plasmid is large and fragile and has a low copy number.

The standard method for preparative purification of plasmid DNA is isopycnic ultracentrifugation through CsCl-ethidium bromide gradients. This method is based on the facts that ethidium bromide (EtBr) can change the density of DNA by intercalating between adjacent base pairs and much more EtBr intercalates in linear DNA than in supercoiled DNA (Pecq 1971). This is the only reliable method presently available to purify Ti plasmids from *Agrobacterium* but it is time-consuming and requires expensive instruments and chemicals.

We observed that in contrast to DNA, EtBr is much more soluble in phenol than in H_2O. It seemed possible that linear DNA with high EtBr intercalation might be soluble in phenol whereas supercoiled plasmid DNA with low EtBr intercalation would remain water soluble. This proved to be so. Here we describe a rapid small scale EtBr-phenol extraction procedure which exploits the differential solubility in phenol of plasmid DNA compared with linear DNA and RNA after EtBr treatment. The method is simple, rapid and efficient and can be scaled up for the preparative isolation of plasmids from litre cultures with quantitative yields.

Materials and Methods

REAGENTS AND BACTERIAL STRAINS

All chemicals were obtained from Sigma or BDH and were at least analytical grade. Solution I (25 mmol l^{-1} Tris, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} glucose, pH 8·0), solution II (0·2 mol l^{-1} NaOH, 1% SDS), solution III (3 mol

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l^{-1} potassium acetate, adjusted to pH 4·8 with glacial acetic acid) and phenol solution (phenol : chloroform : isoamylalcohol, 25 : 24 : 1, containing 0·1% 8-hydroxyquinoline, pH $<7{\cdot}6$) were prepared as described by Maniatis *et al.* (1982). *Agrobacterium tumefaciens* strain A6 and *E. coli* strains HB101 (pVK102) and HB101 (pBIN19) were used in this study.

SMALL SCALE PLASMID ISOLATION AND PURIFICATION PROCEDURE

The small scale isolation procedure described here is based on the modified alkaline procedure of Currier and Nester (1976). Bacteria were grown for 24 h in 10 ml of YEB medium (Van Larebeke *et al.* 1977) at 28°C, collected by centrifugation (11 000 *g*) in 10 ml tubes for 2 min and transferred to a 2 ml tube after resuspension in 1 ml LTE buffer (10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 8·0), 100 μl 5 mol l^{-1} NaCl and 20 μl 10% Na Sarkosyl. The bacterial suspension was mixed gently and centrifuged (11 000 *g*) for 2 min. The pellet was resuspended in 200 μl of solution I containing 2 mg ml^{-1} lysozyme and placed on ice for 5 min. Bacteria were lysed by adding 400 μl of solution II, inverting gently to mix and leaving at 37°C for 10 min. The solution was neutralized by adding 100 μl 2 mol l^{-1} Tris (pH 7·0), mixed well but gently by inversion and then let stand for 30 min at room temperature; 100 μl 5 mol l^{-1} NaCl and 800 μl of phenol solution were added and mixed gently by inversion for 5 min. The aqueous phase was collected and transferred to a fresh 2 ml tube after centrifugation for 10 min. The crude DNA was precipitated by adding an equal volume of isopropanol, mixing and centrifuging for 15 min. The pellet was washed once with 1 ml 70% ethanol and dried in air for 2 min.

For purification, the following EtBr-phenol extraction procedure was used. The pellet was dissolved by adding 100 μl solution I, 170 μl sterile H_2O and 10 μl 2 mol l^{-1} Tris, mixing gently and leaving at 50°C for 5 min. After adding a mixture of 20 μl 2 mol l^{-1} NaOH and 150 μl ice-cold solution III, the solution was left on ice for 5 min. Then 40 μl 1% EtBr was added, mixed gently and left on ice for 30 min; after gentle mixing, the solution was centrifuged for 5 min. The supernatant was extracted using an equal volume of phenol solution by slowly inverting for 5 min. The aqueous phase was collected by centrifugation for 5 min and re-extracted for 3 min with the same volume of chloroform. Plasmid DNA was precipitated by adding an equal volume of isiopropanol, mixing and centrifuging for 15 min. The pellet was washed twice with 70% ethanol and dried *in vacuo*. It was then dissolved in 20 μl LTE. The amount of Ti plasmid DNA in such a solution is sufficient for three restriction enzyme digests.

PREPARATIVE PURIFICATION OF PLASMID DNA

The large scale alkali lysis method (Maniatis *et al.* 1982) and the modified alkali lysis method (Currier and Nester 1976) were followed to isolate plasmid DNA from 1 l cultures of *E. coli* and *A. tumefaciens* respectively. The plasmid pellet precipitated by ethanol was dissolved in 4 ml of solution I, 6·8 ml of sterile H_2O and 400 ml of 2 mol l^{-1} Tris buffer. The dissolved DNA solution was distributed equally into four 10 ml disposable plastic centrifuge tubes. To each tube, 20 μl of 2 mol l^{-1} NaOH solution was added and left at room temperature for 5 min before neutralization with 1·5 ml of solution III. Then the EtBr-phenol extraction procedures described above were followed, using 10 times the amount of EtBr.

EFFECT OF ETHYDIUM BROMIDE ON THE PARTITION OF LINEAR AND PLASMID DNA INTO PHENOL AND AQUEOUS PHASES

The EtBr-phenol extraction procedure described above was followed to study the effect of EtBr on the partition of plasmid DNA and linear DNA into phenol and aqueous phases.

Linear salmon sperm DNA was labelled with ^{32}P-dCTP using a nick translation kit (BRESA) according to the manufacturer's instructions. The radioactivity of ^{32}P-labelled linear DNA was about $1{\cdot}2 \times 10^7$ cpm μg^{-1}. After EtBr-phenol extraction, radioactivity in the phenol and aqueous phases was measured in a Beckman LS 5000 TD Liquid Scintillation System.

Results and Discussion

EFFECT OF EtBr ON THE PARTITION OF LINEAR DNA AND PLASMID DNA INTO AQUEOUS AND PHENOL PHASES

Different sized plasmids purified from *A. tumefaciens* and *E. coli* were used for the assessment of recovery of plasmid DNA in the aqueous phase after EtBr-phenol extraction. Table 1 shows that all denatured plasmid DNA was recovered with an efficiency of more than 90%. In fact, recovery of small sized plasmid DNA was almost 100%. However, following denaturation, in which the supercoiled structure of plasmid DNA was destroyed by heat, about 90% of all plasmid DNA was removed by EtBr-phenol extraction (Table 1).

To determine the effect of EtBr on the partition of linear DNA into aqueous and phenol phases, ^{32}P-labelled salmon sperm DNA was used. Figure 1 shows that EtBr has a marked effect on the partition of linear DNA into the phenol phase. Nearly all linear DNA remained in the aqueous phase if not treated with EtBr before phenol extraction, whereas following 30 min EtBr treatment, 87·4% of linear DNA was present in the phenol phase. Fractionation of the phenol phase indicated that the majority of the ^{32}P-labelled linear DNA was in the top layer of the phenol phase (data not shown).

Table 1. Recovery of plasmid DNA after EtBr-phenol extraction

Plasmid (size)	Percentage recovery* Supercoiled plasmid	Denatured plasmid†
pBIN19 (10 kb)	100	9·8
pVK102 (23 kb)	99·2	9·7
pTiK585 (197 kb)	91·6	9·4

* The experiments were repeated three times, using 24 μg of plasmid DNA for each experiment.

† Plasmid DNA was denatured by heating in boiling water for 10 min, then placing in ice water for 5 min.

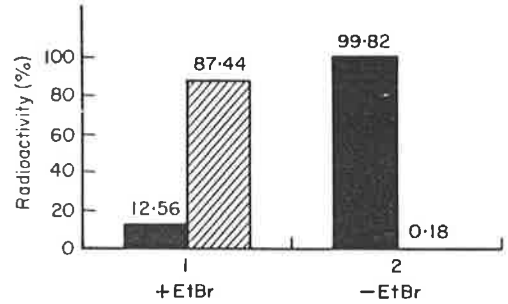

Fig. 1. Effect of ethydium bromide on the partition of linear DNA into phenol (□) and aqueous (■) phases. The experiment was repeated three times using a mixture of 5 μl of ^{32}P-labelled linear DNA (about 20 ng) and 80 μg unlabelled linear DNA.

YIELD AND QUALITY OF PURIFIED PLASMID DNA

Figure 2 shows a comparison of the DNA isolated before and after EtBr-phenol extraction and the restriction enzyme digestion patterns of the purified plasmid DNA; only a trace of genomic DNA and RNA can be detected after EtBr-phenol extraction. Figure 2 also shows that purified plasmid DNA is subject to complete digestion by *Hin*dIII and *Eco*R1 restriction enzymes.

We compared the efficiency of Ti plasmid extraction by the EtBr-phenol protocol and by CsCl–EtBr gradient ultracentrifugation. The yields of Ti plasmid were about 180 μg to 300 μg per l varying from batch to batch after ultracentrifugation. The yield of Ti plasmid by the new method was less variable and much higher,

Fig. 2. Ti plasmid profiles and restriction enzyme digestion patterns. Lanes 1 and 3 are *Hin*dIII and *Eco*R1 digestion patterns respectively of pTi A6 purified by CsCl–EtBr gradient ultracentrifugation; lanes 2 and 4 are the same enzyme digestion patterns of pTi A6 purified by EtBr-phenol extraction; 6 μg plasmid DNA was digested by 3 units of restriction enzyme at 37°C for 30 min. Lanes 5 and 6 show pTi A6 from *Agrobacterium* after and before EtBr-phenol extraction. Chr, Chromosomal DNA.

being around 2500 $\mu g\ l^{-1}$ each run. This procedure has been successfully used in our laboratory for more than 1 year to purify Ti plasmids ranging from 170 to 200 kb for genetic mapping and DNA cloning, with quality comparable to and yield 8 times better than is obtained by EtBr–CsCl density gradient ultracentrifugation.

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