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Construction of a gene bank and use of the chromosome walking technique for the detection of new putative agrocin genes in Agrobacterium tumefaciens strain D286

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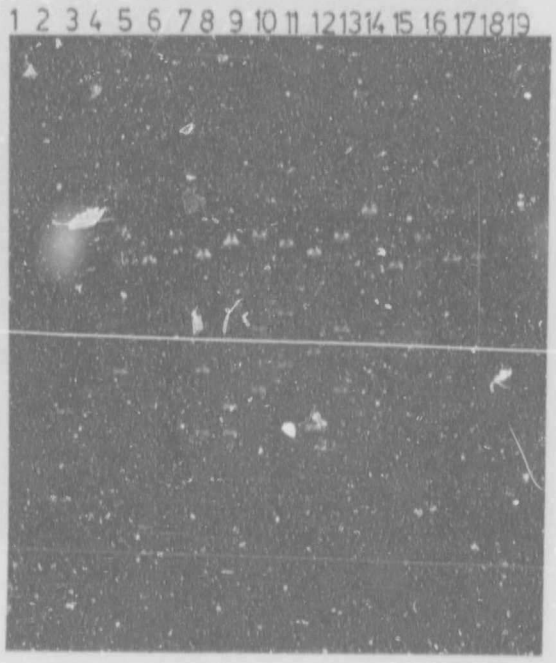


Figure 4.20. Gel electrophoresis (0.45 % agarose; run for 36 hs) of single and double digests of pCD0932 and pCD2375 with EcoRI, SalI and BglII. Lanes 1, 4, 7, 10, 13, 16 and 19, λ H (Sal I). Lanes 2-3, 5-6, 8-9, pCD0932 digested with EcoRI, SalI, BglII, EcoRI + SalI, EcoRI + BglII and BglI + SalI respectively. Lanes 11-12, 14-15, 17-18, pCD2375 digested with EcoRI, SalI, BglII, EcoRI + SalI, EcoRI + BglII and BglII + Sal I respectively.

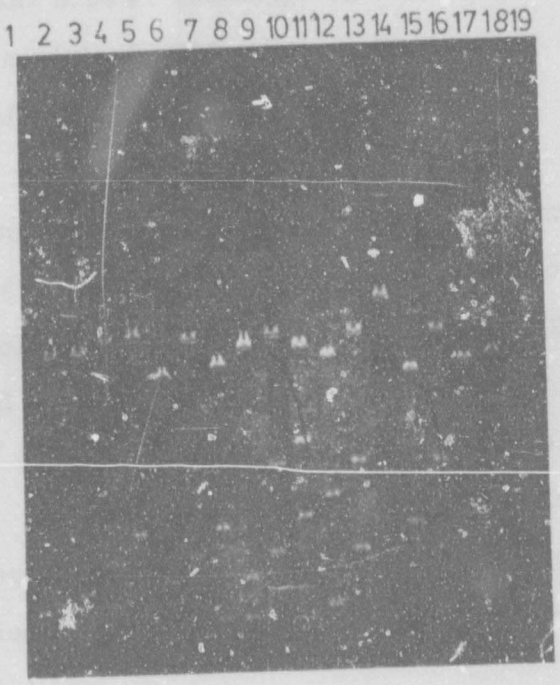


Figure 4.21. Gel electrophoresis (0.45 % agarose; run for 48 hs) of single and double digests of pCD0932 and pCD2375 with EcoRI, SalI and BglII. Lanes 1, 4, 7, 10, 13, 16 and 19, λ H (λ II). Lanes 2-3, 5-6, 8-9, pCD0932 digested with EcoRI, SalI, BglII, EcoRI + SalI, EcoRI + BglII and BglII + SalI respectively. Lanes 11-12, 14-15, 17-18, pCD2375 digested with EcoRI, SalI, BglII, EcoRI + SalI, EcoRI + BglII and BglII + Sal I respectively.

4.6 TRANSFORMATION OF A.TUMEFACIENS C58 C1G WITH PCD0932 AND CONJUGATION OF E. COLI DH1 (PCD0932) X A. TUMEFACIENS D286 : : TN5 AG-

A categoric proof that the D286 wt DNA insert of pCD0932 does indeed carry genes affecting agrocin D286 production can be provided through the restoration of the D286 wt phenotype by complementation of the D286::Tn5 Ag-. mutant strain with pCD0932. With this purpose, it was attempted to conjugate *E. coli* DH1 (pCD0932) x D286::Tn5 Ag-.

In the conjugation experiments the concentration of the antibiotic Tc was varied (10 and 2 µg/ml) but there were no differences between the number of colonies arising in control plates (D286::Tn5 Ag^- plated on NA supplemented with 100 µg/ml Neo, Nal and 10 or 2 µg/ml Tc) and those of conjugation cultures. The average number of colonies of 20 plates inoculated with 200 µl of 10^{-2} dilutions of cultures were 625 and 533 for control plates and 578 and 551 for the conjugation cultures in Tc at concentrations 10 and 2 µg/ml (concentrations of Neo and Nal were constant ; 100 µg/ml).

In order to determine if the 21.8 kb D286 wt DNA insert of pCD0932 contained all the information necessary for the production of agrocin D286 it was attempted to transform the plasmmidless *A. tumefaciens* strain C58 C1G with pCD0932. In this case the only selectable marker ,Tc,in the system was borne by pCD0932 The number of colonies arising from cultures subjected to the transformation protocol with and without (control) pCD0932 DNA was not different.

It was concluded that Tc resistance is not an efficient selectable marker for the systems used in the complementation studies in this work, as is the case for other systems involving *Agrobacterium* strains (Farrand, personal communication). In addition, the frequency of transconjugants

arising using the system in this work is about 10^{-5} (Farrand, personal comunication). It therefore became evident that the screening process for detection of transconjugants would be an exercise with little chance of success in the absence of an efficient selectable marker.

On the above grounds it was decided to subclone the D286 wt DNA insert of pCD0932 into a mobilizable broad-host-range vector with an effective selectable marker which would make possible the rapid selection of transformed and transconjugant *Agrobacterium* strains.

4.6 CONSTRUCTION OF PSUP204-1

pSUP204-1, a derivative of pSUP204 (Priefer et al., 1985) was constructed with the purpose of using it as a subcloning vector conferring resistance to Amp. pSUP204 confers resistance to Amp, Cm and Tc. For our purposes Tc resistance was an undesirable trait (see discussion section) therefore it was eliminated by insertion inactivation. For this a 700 bp λ DNA SalI fragment was cloned into the unique SalI site od pSUP204.

DNA from λ bactericphage (Boehrinheim Manheim) was subjected to total SalI digestion and the sample electrophoresed in a 1% agarose gel. The smallest fragment (700 bp) was then isolated by electroelution. This fragment (20 ng) was ligated to pSUP204 opened at its unique SalI site. The ligation was used to transform *E. coli* SM10 and transformants selected by plating onto LB agar supplemented with Amp (200µg/ml) and Cm (200µg/ml). Transformants were obtained at an efficiency of 1.4×10^4/µg ligated DNA. Of the approximately 1700 transformants obtained, 1000 were screened for loss of resistance to Tc by replica plating them on LB agar + Amp (200 µg/ml) + Cm (200 µg/ml) + Tc (20 µg/ml) and on LB agar + Amp and Cm at the same concentrations as above but with no Tc. Nine colonies resistant to Amp and Cm but sensitive to Tc were detected. Plasmid DNA from one of such colonies was isolated, subjected to SalI digestion and

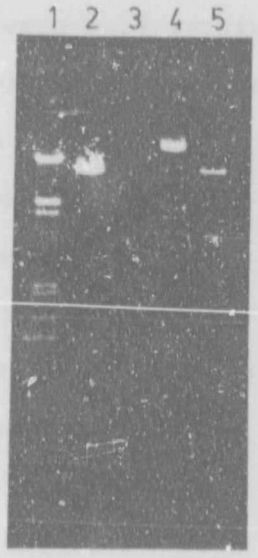


Figure 4.22. Agarose gel electrophoresis of pSUP204-1 undigested and undigested with SalI and with EcoRI. Lane 1, λ HE (λ III); 2, pSUP204-1 digested with SalI; 3, SalI λ DNA fragment used for ligating into SalI restriction site of pSUP204; 4, pSUP2041 undigested; 5, pSUP2041 digested with EcoRI.

electrophoresed in a 1% agarose gel. A SalI fragment migrating in the same position than the λ DNA 700 bp SalI fragment was detected (Figure 4.22) demonstrating that loss of Tc resistance had been brought about by insertion inactivation of the marker gene in pSUP204. The slightly modified pSUP204 was named pSUP2[illegible]-1.

Since after cloning in the EcoRI site of pSUP204-1 only resistance to Amp would remain as a selectable marker, the sensitivity of strains D286::Tn5 Ag^- and C58 C1G to two concentrations of such antibiotic was tested (Table 4.2). Both strains were very sensitive to an ampicillin concentration of 200µg/ml and strain C58 C1G was very sensitive to the lower concentration (100µg/ml) as well. Work is underway to subclone the D286 insert of pCD0932 in pSUP204-1. Since the insert is composed of 4 EcoRI fragments the only way of isolating the entire fragment is through partial EcoRI digestion of pCD0932 and purification of the 21.8 kb D286 DNA fragment. An EcoRI calibration digestion of pCD0932 was carried out to determine optimal conditions for the generation of fragments larger than 20 kb (Figure 4.23) the conditions leading to the restriction pattern observed in Figure 4.23, lane 8 were then used in scaled up digestions. The digested DNA was then electrophoresed and the larger fragments electroeluted and purified. Approximately 40 ng of the isolated DNA were ligated to pSUP204-1 opened at its EcoRI site. The ligated DNA is currently being used in this lab in an attempt to subclone the D286 wt DNA insert of pCD0932 for further use in agrocin D286-production complementation studies.

TABLE 4.2 Ampicillin sensitivity of strains C58 C1G and D286::Tn5-3

	Number of colonies* ($5x10^{-5}$) arising per ml of culture		
Strain	No Amp	Amp(100ug/ml	Amp(200ug/ml)
C58 C1G	1440	0	0
D286::Tn5Ag$^-$	1630	215	0

*Colony numbers were estimated from 10^{-5} dilutions

electrophoresed in a 1% agarose gel. A SalI fragment migrating in the same position than the λ DNA 700 bp SalI fragment was detected (Figure 4.22) demonstrating that loss of Tc resistance had been brought about by insertion inactivation of the marker gene in pSUP204. The slightly modified pSUP204 was named pSUP204-1.

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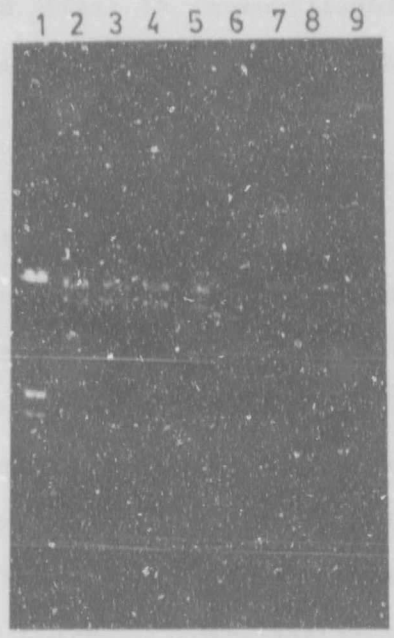


Figure 4.23. Agarose gel electrophoresis of EcoRI calibration digestion of pCD0932 for determination of conditions yielding restriction patterns abundant in large fragments. Digestions were carried out for 1 h at 37° C . Lane 1, λ HE (λ III); 2-9, pCD0932 digested with 5.69, 2.85, 1.42, 0.71, 0.36, 0.18, 0.09, 0.045, 0.00 U EcoRI/µg DNA.

5 DISCUSSION

pLAFRI (Friedman *et al.*, 1982), the vector used for the construction of the D286 wt gene library, was chosen because it offers the following advantages. (i) Being a cosmid vector it allowed the cloning of large DNA fragments, a prerequisite for chromosome walking (Hadfield, 1983). (ii) It is a broad host range EcoRI vector which replicates and is stably maintained in a wide range of Gram negative-bacteria including *E. coli* and *A. tumefaciens* and (iii) it is mobilizable by the helper plasmid pRK2013 into a number of Gram-negative bacteria, including *A. tumefaciens*.

The large average size of the DNA inserts of the D286 wt gene bank (27.7 kb) constructred in this work (an advantageous feature for chromosome walking), was partly due to the cosmid nature of pLAFRI. However, other advantageous features of pLAFKI were masked by its lack of an efficient selectable marker for transformed *A. tumefaciens* strains. The use of Tc resistance (conferred by pLAFRI) as a selectable marker for transformed *A. tumefaciens* strains has been described by Klee *et al.* (1985) as being of limited use. Farrand (personal communication) has experienced different degrees of success using Tc resistance as a selectable marker depending on the *A. tumefaciens* strain. Likewise, in this laboratory the resistance to tetracycline of different strains varies, in the particular case of strains D286 and C58 C1G , transformation and conjugation results indicate that Tc is not a useful selectable marker.

In order to effectively select transformed D286 and C58 C1G cells a vector such as pSUP204 (Priefer *et al.*, 1985) can be used as a subcloning vector. pSUP vectors combine the mobilisation and the broad-host-range replication functions of RSF1010 with the advantages of conventional cloning vectors commonly used in *E. coli*. pSUP204 is based on pBR325 and confers resistance to Tc, Cm and Amp. Since the D286 DNA inserts of pCD0932 and

pCD2375 contain internal EcoRI restriction sites their isolation must be carried out from electrophoresed partial EcoRI digestions. In this work digestion conditions for the generation of a restriction pattern abundant in large fragments were established. However, the D286 DNA inserts in pCD0932 and pCD2375 are 21.7 and 24.8 kb respectively and pLAFRI is 21.6 kb which is conducent to contamination of D286 DNA fragments with pLAFRI during their isolation from agarose gels for subsequent subcloning. To overcome this problem we eliminated the Tc resistance marker of pSUP204 resulting in pSUP204-1, which confers only Cm and Amp resistancce. In this manner pSUP204-1 hybrids harboring a pLAFRI insert will be resistant to Tc whereas those with D286 inserts will not. Cloning into the EcoRI site of pSUP204-1 eliminates resistance to Cm but in this work it was shown that strains D286 and C58 C1G are very sensitive to Amp (100 µg/ml). Therefore after mobilisation into D286::Tn5 Ag^- the hybrid pSUP204-1 plasmids should be easily detected by selection on Amp containing rich medium.

In the construction of pSUP2041 it was chosen to clone the 700 bp SalI λ DNA fragment into pSUP204 because such fragment is (i) relatively small, (ii) it does not contain restriction sites borne by pSUP204 and (iii) because experience indicates lack of homology with DNAs commonly used in this laboratory (most Southern blotted gels include λ DNA molecular weigh markers), therefore it will not cross-hybridise with probes commonly used in this lab. An advantage of insertion inactivation of the Tc resistance gene over, for instance, filling in the ends of SalI digested pSUP204 and blunt end ligation is that the latter approach does not regenerate the SalI restriction sequences whereas the SalI site remains available in pSUP204-1.

During chromosome walking it was only possible to take one step from the initial detection of pCD0932 using pCDTn5-3 to the detection of pCD2375 using pCD0932. Since pCD0932 and pCD2375 are 21.8 and 24.8 kb respectively

and they share a common 3.5 kb fragment they cover a 43.1 kb D286 wt DNA fragment. The detection of the DNA fragment flanking the remaining side of pCD0932 will be facilitated if only the EcoRI fragment in the end opposite to the pCD0932-pCD2375 overlapping sequences is used as a probe. This requires the characterization of pCD0932 and pCD2375 D286 DNAs by restriction endonuclease digestions. In addition, if the entire D286 DNA of pCD0932 plus its flanking sequences detected through chromosome walking is to be used in further genetic manipulations it will be necessary to ligate the three fragments and restriction digestion characterizations will be necessary to assess whether they have ligated in the correct orientation.Also, as it has been discussed elsewhere in this section, a reasonably detailed restriction map of the D286 DNA inserts in pCD0932 and flanking fragments is critically important if site directed mutagenesis of D286 wt is undertaken.

With this purpose the construction of an EcoRI, SalI ,BglII restriction map of pCD0932 and pCD2375 was started. Electrophoresis conditions have been established in order to discriminate size differences among fragments larger than 20 kb. However, it appears that isolation of the entire D286 DNA insert from the hybrid cosmids for subsequent restriction digestions will be necessary in order to eliminate ambiguities. In this regard use of the subcloning vector pSUP204-1, will facilitate the isolation of entire D286 inserts product of partial EcoRI digestions,due to its relatively small size (12 kb)

Conclusions and parting remarks

As a result of the present work 2 cloned D286 DNA fragments, at least one of them bearing putative agrocin genes (pCD0932), covering a region of 43.1 kb are now available for further manipulations aimed at detecting agrocin genes. Preliminary restriction digestions results indicate the

necessity to isolate the entire insert DNAs from pCD0932 and pCD2375 in order to eliminate ambiguities in the restriction patterns.

Failure to detect transformed colonies during transformation and conjugation involving strains C58 C1G and D286::Tn5 Ag- are attributable to Tc resistance not being an efficient selectable marker. pSUP204-1, constructed in this work, should prove useful as a subcloning vector for transformation and conjugations involving *Agrobacterium* strains because it conferrs resistance to Amp , to which *Agrobacterium* strains D286 and C58 C1G proved to be very sensitive.

Characterization of the cloned D286 DNA fragments related by homology and contiguity to the D286 DNA sequences in pCDTn5-3 should be carried out. Complementation of agrocin production to restore the wt phenotype of strain D286::Tn5 Ag- and restriction mapping characterization of the fragments is necessary.

Future work will be aimed firstly at determining the extent of the chromosomal DNA carrying genes involved in agrocin production and secondly to cloning such DNA in an *Agrobacterium* expression vector to determine if it contains sufficient information to direct agrocin production. For the first objective, site directed mutragenesis could be carried out. The approach, similar in principle to that of Comai *et al.* (1983), basically involves (i) the subcloning of the D286 DNA insert in the pCD hybrid cosmid in a vector which can be mobilized but can not replicate in *A. tumefaciens*. (ii) The hybrid vector is then subjected to Tn5 mutagenesis and clones are selected that bear Tn5 in different regions of the inserted DNA. (iii) Such recombinant vectors are then mobilized into D286 wt and (iv) selection for kanamycin resistance should yield co-integrates and mutations which can be discriminated by screening for loss of a selectable marker present in the cloning vector. In this manner loss of agrocin production can be correlated to a particular mutation,

the exact location of which, within an existing cloned D286 DNA fragment can be determined by analysis of restriction patterns Analysis of several mutants will be useful in determining the fraction of the cloned DNA involved in agrocin D286 production. Such fragment can then be reconstructed from the existing cloned D286 DNA, cloned into an adequate expression vector for *Agrobacterium* strains (such as pNM185; Mermod *et al.*, 1986) and once introduced into a plasmidless Ag- *Agrobacterium* strain , agrocin D286 production assessed.

The fact that only one D286::Tn5 Ag- mutant is available is not encouraging. If plasmid curing of D286 wt, up to now unsuccessful, results in loss of agrocin production , plasmid mutants will have to be generated in order to elucidate the molecular biology of agrocin D286 genes.

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