In an attempt to find agrocins with a broader host range than that of the very specific agrocin 84, Hendson *et al*., (1983) screened strains isolated from crown galls on a variety of plants. They isolated numerous agrocin producing strains, most of them with very limited host ranges. However, one of the strains; the non-pathogenic *A. tumefaciens* strain D286, produced an agrocin with a broader host range than agrocin 84. Hendson *et al*. found that sensitivity to agrocin D286 maped in a region that falls within one of the regions homologous to both pTiC59 and the octopine plasmids pTiB653and pTiAch5 and may explain the broad host range of agrocin D286.

Breakdown of biological control of crown gall can occurr in two manners. The first one involves the transfer of pAgK84 (which encodes agrocin synthesis; Cooksey and Moore, 1981) by pAt84b (which carries genes for mobilizing pAgK84; Ellis *et al*., 1979) to a pathogenic strain. The second mechanism of breakdown is by the transfer of a Ti-plasmid from a pathogenec strain to strain 84. This could be prevented by transferring the agrocin encoding plasmid to a bacterium unlikely to receive and/or maintain a Ti-plasmid. Using this approach Hendson and Thomson (1986) transferred the plasmid encoding agrocin production from *A. tumefaciens* 396 to *Rhizobium meliloti*. The agrocin plasmid was stably maintained and produced yields of agrocin comparable to the parental strain.

From the biological control point of view it is important to determine where the agrocin genes of a particular strain map and to analyze such DNA. This can be useful in determining the vulnerability of the strain to the two mechanisms of breakdown of biological control and in designing a system to overcome it. For instance, a strain harboring the agrocin production genes on its chromosome (as appears to be the case of *A. tumefaciens* D286) is unlikely to be affected by the first mechanism of breakdown and if the agrocin genes are to transferred to another strain (to avoid the second mechanism of breakdown and/or to capitalize on ad-

vantages that may be conferred by a different host strain) detailed knowledge of their position and organization is required in order to clone them into adequate delivery vehicles. Thomson (1986) points out in connection with biological control af crown gall in vines (not yet possible) that with the advent of genetic engineering, we are no longer reliant on the organisms that nature provides: we can now tailor our biocontrol strains to fit the disease. If the genes coding for the broad host range agrocin D286 are identified and cloned, they can be cloned into organisms with outstanding colonization characteristics, providing in this manner a means of biological control of crown gall in some plants where agrocin 84 has proven ineffective.

Research on strain D286 is currently being carried out in the CSIR, LMCB.In order to localize the agrocin D286 producing genes Botha and Thomson (unpublished data) utilized transposon mutagenesis, selection for drug resistance, and hybridisations in the following manner. Transposon mutagenesis was carried out by conjugating *E. coli* SM10 (pSUP1011) with strain D286. Since Tn5 (in vector pSUP1011) carries genes for resistance to neomycin, transconjugants were detected by selection for neomycin resistance. Screening of the neomycin resistant strains for loss of the capacity to produce agrocin resulted in the identification of D286::Tn5 Ag- mutants. Only one of the mutants maintained its phenotype. Total DNA from the D286::Tn5 Ag- mutant was subjected to partial BamHI restriction digestion and the resulting fragments were cloned into the BglII site of PSUP106 (Priefer *et al.*, 1985) in *E. coli*. This was followed by selection for neomycin resistance. Nine neomycin resistant clones were obtained; they were called pCDTn5-1 to 9.

Since the gene bank had been made with D286::Tn5 Ag- DNA, the neomycin resistant clones had D286 DNA which coded at least for part of the functions involved in agrocin D286 production. Therefore DNA homology of pCDTn5 (1)-(9) with D286 wt DNA should provide a means of detecting D286

wt agrocin D286 genes. With this purpose Botha and Thomson (unpublished work of this laboratory) constructed a D286 gene bank in the cosmid vector pLAFRI (Friedman *et al.*, 1982). They cloned large fragments, 20-30 kb, from a partial D286 wt DNA EcoRI digestion into the unique EcoRI site of pLAFRI. A ^{32}P labelled pCDTn5-2 probe was then used to probe the D286 wt genomic library using the *in situ* colony hybridisation method (Grunstein and Hogness, 1975). In this manner one positive clone was detected; it was called pCD1253.

The selection of clones carrying putative agrocin D286 genes in the D286 wt library has thus far met with limited success because it has been possible to isolate only one Ag- mutant (D286::Tn5 Ag-). However, Farrand *et al.* (1985) found that a single 20kb fragment of the agocin 84 encoding plasmid pAgK84 is responsible for the production of agrocin. If the production of agrocin D286 is also determined by a DNA fragment of similar size it follows that the D286 DNA sequences that can be detected through homology with the existing pCDTn5 (1)-(9) will contain at least part of the information necessary for agrocin D286 production. Transfer of the hybrid vectors containing putative agrocin D286 genes into a plasmidless *Agrobacterium* strain such as C58 C1G and assessing if the strain produces agrocin 84 would be a categoric demonstration that the DNA sequences containing the information necessary for agrocin D286 production have been cloned. It is possible, however, that only part of the DNA encoding agrocin D286 has been cloned. In that case, working on the assumption that agrocin D286 production is encoded in a single , continuos DNA stretch the totality of the DNA sequences can still be detected using the chromosome walking technique .

Chromosome walking is a technique which utilizes the unique sequence of an initial cloned fragment of chromosomal DNA to identify, by homology, a set of genomic library clones that contain all or part of the initial clone's sequence and also extend into the adjacent DNA along the chrom-

some (Hadfield, 1983). The identification of this cloned adjacent DNA represents a single walking step. The chromosomal walk is continued by another similar walking step. This leads to the isolation of a further set of overlapping genomic library clones, which extend the cloned region further down the chromosome. Such walking will occur in both directions along the chromosome from the starting position. Chromosome walking provides a means of cloning the regions adjacent to an existing clone. Starting with a fragment of a gene or cDNA, the entire gene, including control sequences can be isolated from the genomic library.

Farrand *et al.* (1985) have located the determinamnts for transfer and agrocin 84 production in plasmid pAgK84. They analyzed the sites and effects of Tn5 insertions in order to construct a functional map of pAgK84. They analyzed, by endonuclease restriction mapping a total of 43 insertions affecting agrocin 84 production, suggesting that there are at least three plasmid regions involved in agrocin 84 biosynthesis. A similar analysis of agrocin D286 production in strain D286 would be practically impossible because restriction mapping would have to be carried out on the chromosomal DNA, where the agrocin D286 genes appear to map. In addition, we only have one D286::Tn5 Ag- mutant which renders the task theoretically impossible. An alternative is to use site directed mutagenesis. This approach (described in the discussion section) basically involves subcloning D286 DNA fragments containing putative agrocin genes (as those detected through chromosome walking) in a vector which can be mobilized but does not replicate in *A. tumefaciens*. Tn5 mutagenesis is then carried out in the hybrid vector in order to label different regions of the insert DNA with a Tn5 sequence and the hybrid vectors mobilized into *A. tumefaciens* D286 wt. Selection on rich medium supplemented with the antibiotic for which the Tn5 marker codes resistance results in detection of mutated D286 clones which have acquired Tn through recombination with the homologous DNA fragment carried in the hybrid vector. Screening such D286 mutants for loss of agrocin D286 production

and determinati of the position of Tn5 in the insert DNA fragments giving rise to D286 Ag- mutants results in the detection of different r ons involved in agrocin D286 production.

The analysis of DNA fragments carrying putative agrocin nes by restriction endonuclease mapping is a critical prerequisite o e above-described approach for the location and characterization of the determinants involved in agrocin D286 production.

3 MATERIALS AND METHODS

3.1 BACTERIA AND PLASMIDS

The bacterial strains and the plasmids used in this study are described in Table 2.1.

3.2 BACTERIAL CONJUGATIONS AND TRANSFORMATIONS

3.2.1 TRANSFORMATION OF E. COLI SM10

E. coli SM10 was tranformed following procedures by Mandel and Higa (1970). A 1:50 dilution of an overnight culture in LB medium (to give a final volume of about 40 mls) was grown at 37° C to an O.D.(λ 600nm) of 0,25 after which the cells were pelleted by centrifugation at 5000 rpm for five minutes. The cells were resuspended in one half the culture volume of 0,05 M $CaCl_2$ and kept at 0° C for fifteen minutes. The cells were pelleted again and resuspended in one tenth the culture volume of 0.05 M $CaCl_2$. Two hundred microliters of cell suspension were mixed with 100 µl of DNA solution. The DNA solution should be in 10 mM Tris pH 7,5; 10 mM $CaCl_2$ 10 mM $MgCl_2$. The cell/DNA mixture was incubated for fifteen minutes at 0°C followed by a ten-minutes incubation at 25°C. In order to allow expression of antibiotic resistance genes 0,5 ml of LB medium was added to each tube and the tubes incubated at 37° C for thirty minutes. Two and a half mls of molten 0,8% agar held at 45° C were then added and mixed on a vortex mixer. The mixture was poured onto LB agar plates containing Amp (200µg/ml) and Cm (200µl/ml). The plates were then incubated for 24 hrs at 37° C. This resulted in two sizes of colonies since the surface bacteria grew faster than those submerged in the agar.

3.2.2 TRANSFORMATION OF A. TUMEFACIENS C58 C1G

TABLE 2.1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source or reference
A. Tumefaciens		
D286 wt	Produces the wide-host-range agrocin D286	Department of Plant Protection South Africa
D286::Tn5 Ag^-	Tn5-generated Ag^- mutant; Km^r	C.Botha, this laboratory
C58 ClG	Plasmidless strain	Hynes *et al*. (1985)
E. coli		
DH1	Plasmidless strain; Rec A^-; inducible λ phage receptors	Massachussets Institute of Technology
DF 3046	Contains pRK2013	S. Farrand U. of Champaigne Urbana. Chicago USA
SM10	Rec$^-$ derivative of C600 with RP4-2::Mu integrated	Simon *et al*. (1983)
Plasmids and cosmids		
pRK2013	Km^r; self transmissible derivative of RK2 containing ColE replicon and tranfer functions necessary to mobilize pRK290 and its derivatives	Figurski and Helinsky (1979)
pSUP204	(12 kb) Ap^r, Cm^r, Tc^r, mobilizable by Inc C-plasmids	Simon *et al*.(1983)
pSUP204-1	(12.7 kb) Ap^r, Cm^r, mobilizable by Inc C plasmids	This work
pLAFRI	(21.6 kb) derivative of wide-host-range vector pRK290, mob^+ tra^+, mobilizable by pRK2013, Tc^r	Friedman *et al*. (1982)
pCDTn5-3	a hybrid cosmid pSUP106-D286::Tn5	C.Botha,this laboratory
pCD1253	a hybrid cosmid pLAFRI-D246 wt DNA, insert size 5kb; Tc^r	C.Botha,this laboratory
pCD0932	a hybrid cosmid pLAFRI-D286 wt DNA, insert size 21.8 kb; Tc^r	This work
pCD2375	a hyorid cosmid pLAFRI-D266 wt DNA, insert size 24.8 kb; Tc^r	This work

TABLE 2.1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source or reference
A. Tumefaciens		
D286 wt	Prod [illegible]-range agrocin D286	Department of Plant Protection South Africa
D286::Tn5 Ag$^-$	Tn5-[illegible]nt; Km^r	C.Botha, this laboratory
C58 C10	Plasm.	Hynes et al. (1985)
E. coli		
DH1	Plasmidless strain, Rec A$^-$; inducible λ phage receptors	Massachussets Institute of Technology
DF 3046	Contains pRK2013	S. Farrand U. of Champaigne Urbana. Chicago USA
SM10	Rec$^-$ derivative of χ600 with RP4-2::Mu integrated	Simon et al. (1983)
Plasmids and cosmids		
pRK2013	Km^r; self transmissible derivative of RK2 containing ColE replicon and tranfer functions necessary to mobilize pRK290 and its derivatives	Figurski and Helinsky (1979)
pSUP204	(12 kb) Ap^r, Cm^r, Tc^r, mobilizable by Inc C-plasmids	Simon et al.(1983)
pSUP204-1	(12.7 kb) Ap^r, Cm^r, mobilizable by Inc C plasmids	This work
pLAFR1	(21,6 kb) derivative of wide-host-range vector pRK290, mob$^+$ tra$^+$, mobilizable by pRK2013, Tc^r	Friedman et al. (1982)
pCDTn5-3	a hybrid cosmid pSUP106-D286::Tn5	C.Botha,this laboratory
pCD1253	a hybrid cosmid pLAFR1-D286 wt DNA, insert size 5kb; Tc^r	C.Botha,this laboratory
pCD0932	a hybrid cosmid pLAFR1-D286 wt DNA, insert size 21.8 kb; Tc^r	This work
pCD2375	a hybrid cosmid pLAFR1-D286 wt DNA, insert size 24.8 kb; Tc^r	This work

transformation of *A. tumefaciens* C58 C1G was attempted as follows. An overnight culture of *A. tumefaciens* C58 C1G grown in LB medium was diluted to give a lightly turbid suspension in a total volume of 20 mls and grown at 27° C to medium turbidity. The suspension was concentrated 100 fold by centrifugation at 10000 rpm for 5 minutes at 4° C and washed in 10 ml 10 mM Tris pH 7.0. The latter was decanted and the cells resuspended in 0,2 ml of LB medium. One hundred microliter-aliquotes of cell suspension were dispensed into cold 10 ml tubes and to each 50 µl of a pCD0932 DNA solution (50µg/µl) was added. The tubes were then placed on a mixture of dry ice and ethanol (-45° C) for 5 minutes, exposing the cells to a freeze shock. This was followed by static incubation at 37° C (heat shock). Following the heat shock 1 ml of LB medium was added to each tube and incubated at 27° C for expression of antibiotic resistance genes. One hundred microliter-aliquotes of the cell culture were plated on LB agar supplemented with Tc (5µg/ml) and incubated at 27° C until the appearence of conspicuous colonies (72 hs).

3.2.3 CONJUGATION OF E. COLI DH1 (PCD0932) X A. TUMEFACIENS D286::TN5 AG-

The hybrid cosmid pCD0932 in *E. coli* DH1 was mobilized into *A. tumefaciens* D286::Tn5 Ag^{-} using plasmid pRK2013 in *E. coli* DF3046 as mobilizer. The method of Simon *et al.* (1983) as implemented by Farrand (U. Champaigne, personal communication) was followed. Overnight cultures of the donor, recipient, and mobilizing strains in nutrient broth were used. Twenty µl of each culture were mixed in each of 20 Eppendorf microfuge tubes and the mixture incubated overnight at 27° C. The mixed culture was then spotted (20µl dots) on nutrient agar plates and incubated for 48 hrs. The resulting colonies were scraped off with a sterilized spatula and the cells suspended in 2 mls of saline solution (0.6% Na Cl). Ten and one hundred fold dilutions of the suspension were made and plated on nutrient agar supplemented with Neo (100 µg/ml), Nal (100 µg/ml) and Tc (2µg/ml).

The plates were incubated at 27° C until the development of conspicuous colonies (about 72 hrs). The plates were compared with control plates where only *A. tumefaciens* D286::Tn5 Ag^- had been plated and incubated under the same conditions than those of the conjugation mixture. This was done in order to decide if screening for agrocin production would be feasible.

3.3 EXTRACTIONS OF DNA

3.3.1 CELLULAR OR TOTAL DNA EXTRACTION

Cellular or total DNA was extracted from *A. tumefaciens* D286 following a procedure by Thomson (CSIR LMGB). An overnight culture of *A. tumefaciens* D286 in 50 ml of LB medium was centrifuged at 10000 rpm for 10 minutes at 4° C and the pelleted cells were resuspended in 1,5 ml of a 50 mM Tris; 100 mM EDTA; 20% sucrose solution pH 8.0. Three hundred and sixty microliters of fresh lysozyme solution in 0.25 M Tris pH 8.0 were added and the mixture incubated on ice for 20 minutes. Four µl of heat inactivated RNAse A (20 µg/µl), 2 µg of proteinase K in 1 µl of 0.25 M tris pH 8.0 and 2 ml of 1% N-Lauryl sarcosine in 75 mM EDTA pH 8.0 were then added and the cell suspension incubated at 37° C for 6 hs. Following incubation 0.945 grams of cesium chloride were added per ml of solution. The mixture without the addition of ethidium bromide was centrifuged at 45000 rpm for 17 hs at 4° C in a Beckman L8-M ultacentrifuge using a Beckman VTi6S.2 vertical rotor. After centrifugation the DNA was recovered by puncturing and dripping the centrifuge tubes through the bottom. The more viscous fraction, containing the DNA, was collected in an Eppendorf tube and the remaining fractions discarded.

3.3.2 EXTRACTION OF PLASMID DNA

Plasmid and cosmid DNAs were extracted using the procedures published by Clewell and Helinsky (1969) and Irsh-Horowicz and Burke (1981).

The Clewell and Helinsky method.

The clewell and Helinsky method was implemented without modifications.

The Ish-Horowicz and Burke method

The "miniprep" method for analytical DNA extractions published by Irsh-Horowicz and Burke (1981) was used as a rapid method for extraction of plasmid DNA. Some modifications were made in the late steps of the method, namely during the precipitation of the DNA.

One volume of propan-1-ol was added to the supernate istead of the 2 volumes of ethanol specified in the original protocol. The supernate was then incubated at room temperature for 15 minutes and centrifuged in an Eppendorf microfuge to precipitate the plasmid DNA. The pellet was washed with 70% ethanol and dried in a Savant Speed Vac vacuum concentrator. The dried DNA was redisolved in 30 µl of TE buffer pH 8.0. Previous to restriction digestions the DNA was cleaned with phenol following Maniatis *et al.* (1982).

3.4 RESTRICTION ENDONUCLEASE DIGESTIONS OF CELLULAR AND PLASMID DNA

3.4.1 PARTIAL DIGESTS

Calibration digestions were carried out to establish adequate partial digestion conditions. A solution containing a known amount of DNA (gen-

erally about 10 µg) was made in restriction enzyme buffer in a final volume of 150 µl. To the first of 9 tubes a 30 µl volume of the DNA solution was added, each of the remaining 8 tubes received a 15 µl aliquote of DNA solution. Five U of the restriction endonuclease EcoRI in a volume of 1 µl were added to tube number 1 and mixed gently. From tube number 1, 15 µl were transferred to tube number 2 and mixed gently thus diluting the enzyme concentration two-fold. The procedure was repeated with the remaining tubes except for tube number 9 which received no enzyme. Thus, tube number 1 contained 2.5 U of EcoRI and the concentration in tubes 2 to 8 decreased in two-fold steps until it reached 0.020 U in tube number 8. Tube number 9 served as an undigested control. The tubes were incubated at 37° C for exactly 1 h and the incubation stopped immediately by the addition of EDTA to 20 mM. The samples were analyzed by gel electrophoresis and the conditions for scaling-up the digestions chosen.

3.4.2 SINGLE AND DOUBLE TOTAL DIGESTS

Medium and high salt buffers were prepared as 5X and 10X stocks respectively, according to Maniatis *et al.* (1982). The volumes used varied between 10 and 40 µl depending on the amounts of DNA needed and the concentration of the samples; In general about 2-4 U enzyme were used per µg of DNA digested. Incubation (at 37° C in all cases) were carried out for 2.5 hs at the end of which the reaction was stopped either by addition of EDTA to 20 mM or by heating the tubes at 65° C for 10 minutes in a water bath.

The above conditions were observed during single digests whereas in double digests the following alterations were made. Digestions were made in a final volume of 40 µl. In the case of restriction endonucleases sharing the same salt buffer requirements the digest was carried out using only one of the enzymes for 90 minutes after which the solution was heated at

65° C for 10 minutes. The second enzyme was then added, in a volume of 1 µl, and digestion carried out at 37° C for a further 90 minutes.

The restriction endonucleases used in this work required either high or medium salt buffers. Therefore, in double digestions with enzymes requiring different restriction buffers, the DNA was first digested (90 minutes at 37° C) with the medium salt buffer-enzyme in a volume of 30 µl. After heating the tubes at 65° C for 10 minutes, 1 µl of 10X medium salt buffer was added, followed by the addition of an appropriate volume of 1 M NaCl and 1 M Tris pH 7.4 in order to increase the salt concentration of the buffer from medium to high. The high salt buffer enzyme was then added, in a volume of 1 µl and the final volume adjusted to 40 µl with distilled water. Incubation was carried out at 37° C for another 90 minutes.

3.5 AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis was carried out using horizontal slab gel apparatus of 2 sizes, the smaller tanks taking gels 6 x 8 cm while the larger tanks took gels of about 16 x 16cm. Electrophoresis in the smaller tanks was generally carried out at 55-70 V and room temperature under which conditions each run took 2-3 hs to complete. Electrophoresis of the large agarose gels were carried out also at room temperature but were run overnight at about 40 V. Agarose gels routinely used were 0.7% agarose in TBE buffer. To both the agarose solution and the TBE running buffer, ethidium bromide (in a 10 mg/ml water solution) was added to a final concentration of ½ µg/ml. When it was necessary to separate DNA fragments larger than 15-20 kb lower concentration agarose gels (0.45-0.5%) were used.

3.6 PURIFICATION OF DNA FRAGMENTS FROM AGAROSE GELS

DNA fragments were purified from agarose gels using a IBI model UEA electroelution unit. The electroelution apparatus is basically a horizontal electrophoresis unit consisting of 2 buffer chambers communicated by a number of perpendicular "V" tunnels of small diameter (about 3 mm). One hundred µl of a 7.7 M NH_4 acetate solution with a few bromophenol blue crystals to aid visualization are dispensed in each of the "V" tunnels to be used taking care to obtain a sharp interfase. The piece of agarose gel containing the DNA of interest is then cut with a clean scalpel (trimming all the extra gel) and positioned on a small receptacle communicating with the "V" tunnel in its region closest to the anode. Electroelution of the DNA fragment is then carried out at 100 V for 15 minutes. The electroeluted DNA remains in the 7.5 M NH_4 acetate solution. Following electrophoresis the 7.5 M cushion buffer is removed (400 µl are pippeted out of each tunnel in order to ensure total removal of electroeluted DNA). The DNA solution is then washed twice with 2 volumes of n-butanol to remove ethidium bromide as well as the bromophenol blue. The DNA is then either precipitated with propan-1-ol or ethanol following Maniatis *et al.* (1982) and redissolved in TE buffer or the NH_4 acetate salt is replaced by TE buffer using a Centricon™ microconcentrator 30 system (Amicon). The centicon microconcentrators basically consist of two joined tubular chambers separated by a low-adsoptive filter membrane (Marashi *et al.*, 1985). The sample, in a volume of up to 2 ml, is placed in one of the chambers and the unit centrifuged in an angle rotor. In this work, using a Beckman JA 20 rotor, a deadstop volume of 40 µl was obtained after centrifugation at 6500 rpm for 15 minutes at room temperature. The sample can be washed repeatedly by the addition of 2 ml of the adequate buffer. In this work it was found that electroeluted DNA washed three times in this manner was satisfactory for ligation and nick translation. Once the NH_4 acetate solution is replaced by TE buffer the collecting chamber is discarded and the unit inverted and centrifuged at 6500 rpm

for 15 minutes in order to collect the 40 µl of sample in a plastic tube provided by the manufacturer. In this work this method has proven highly efficient, in agreement with other authors (Marashi *et al.*, 1985) who reported DNA recoveries higher than 95%.

3.7 LIGATIONS

Ligations were carried out in 66 mM Tris pH 7.6, 6.6 mM NaCl; 10 mM dithiothreitol; 1 mM ATP pH 7.0 using T4 DNA ligase. Ligation mixtures were incubated overnight at 10° C after which reactions were stopped by heating at 65° C for 5 minutes or by the addition of EDTA pH 8.0 to 20 mM. EDTA was added when it was necessary to store ligations at -20° C. The ligation volumes were kept as low as possible but this depended on the concentration of the vector and insert DNAs. In general vector:insert molar ratios 1:2 and 1:5 were used, provided sufficient DNA was available. Absolute amounts of approximately 0.5-1 mg of vector DNA were used.

3.8 DNA HYBRIDISATIONS

^{32}P-labelling of probes

The various DNA preparations used as probes in this work were labelled with [α-^{32}P]dCTP using an Amersham nick translation kit. The manufacturer's instructions were followed. The amount of DNA labelled varied from about 300 ng (recombinant cosmids pCD1253 and pCD0932) to about 1 µg (the remaining probes). The reactions were carried out in a final volume of 100 µl. Incorporation rate was monitored in 1 µl aliquotes taken at 15 minute intervals. Such aliquotes were spotted on a nitrocellulose paper (about 1 cm^2) and dried on the bench. The desintegrations per second were then quantified with a Geiger-Muller counter (total counts). The sample was counted again after being fixed with 10% TCA for 10 minutes on ice and washed twice with 5% TCA. The latter dpm represent incorporated label.

The reaction was stopped with 5 µl of 0.2 M EDTA when the incorporated-label dps leveled off. Unincorporated [α-^{32}P]dCTP was removed using a spin column (Maniatis *et al.*, 1982). One microliter of the probe was added to 10 ml of scintillation cocktail (Insta-Gel, Packard) and the sample counted in a Packard Minaxi Tri-carb 4000 scintillation counter for 1 minute. The specific activity of the labelled probe was then determined in order to optimize its use during hybridisations.

DNA denaturation

Gels containing DNA of interest were left on a UV transilluminator (UVP inc.) for 10 minutes after which DNA strand separation was achieved by gently shaking in a 1 M NaCl-0.5 M Na OH solution for 30 minutes at room temperature. The gel was then rinsed in water for 10 minutes and neutralized by washing it twice for 30 minutes in a 3 M NaCl-0.5 M Tris pH 7.0. Neutrality was checked with pH paper (Merck). The gel was then soaked in 10x SSC for 10 minutes.

Southern blotting

DNA transfer to presoaked (10x SSC) nitrocellulose paper (Millipore) was carried out overnight at room temperature, following the method published by Southern (1975). The gels were then removed, stained in a 2 µg/ml ethidium bromide solution and DNA transfer checked by viewing in a UV transilluminator. The nitrocellulose paper was then soaked in 4x SSC for 10 minutes, dried at room temperature and baked under vacuum (stappled in a blotting paper envelope) at 80° C for 2 hs.

Preparation of NTC paper-colony filters for *in situ* colony hybridisations

A modification of the Grunstein and Hogness (1975) filter screening procedure was used as follows. Colonies to be tested were patched (by stabing with a sterile toothpick) on LA plates and grown overnight at 37° C. The colonies were then transferred to NTC paper filter (0.25 µm pore size) by applying the filter on top of them and lifting it smoothly. The filters, with the colonies attached to them, were then laid on fresh LA plates, colony sides up, and incubated overnight at 37° C. The following steps were performed by placing the filters on blotting paper saturated with the approppiate salts. Filters were placed, colony sides up, on 0.5 N NaOH for 7 minutes and then transferred to 1.0 M tris-HCl pH 7.4 for 2 minutes. The filters were transferred for a second time to 1.0 M tris-HCl pH 7.4 for a further 2 minutes. The filters were then transferred to 1.5 M Na Cl, 0.5 M tris-HCl pH 7.4 for 4 minutes after which they were dried on blotting paper and baked under vacuum fo 1 h at 80° C..

Hybridisations

Prehybridisations and hybridisations were carried out in formamide solution basically as described by Mason and Williams (1985), at 42° C. Instead of using plastic bags for the prehybridisation and hybrydisations, plastic Tupperware containers were used. During *in situ* colony hybridasations, to avoid the problem of filters sticking together and breaking, dispossable plastic Petri dishes were used. Two filters could be hybridised together (colony sides facing each other) using a minimal amount of hybridisation mixture (3-7 ml). This approach resulted in the filters being evenly exposed to the prehybridisation and hybridisation mixtures.

During hybridisations, labelled probes were added in a ratio of 10^5 cpm/ml hybridisation mixture when the probes had a specific activity of

about 1×10^8 cpm/µg DNA. With probes labelled at a lower specific activity the ratio was increased two or three-fold.

Autoradiography

Filters sealed in platic bags were placed in a X-ray cassette with KodaK XAR-5 X-ray film and exposed overnight at -70° C. After developing, if necessary the exposure time was adjusted and another X-ray film exposed.

3.9 IN VITRO PACKAGING OF PLAFRI-D286 DNA LIGATIONS AND INFECTION OF E.COLI DH1

A λ DNA *in vitro* packaging kit (Amersham) was used following the manufacturers instructions. The resulting phage particles were used to infect the plasmidless *E. coli* DH1 (a *sup* F strain). The antibiotic marker of pLAFR1, Tc, was then used to select for infected clones (carrying pLAFR1-D286 DNA). Only such clones developed as conspicuous colonies on LA plates supplemented with Tc (20 µg/ml).

3.10 SOURCES OF REAGENTS AND CHEMICALS

Agar technical	Oxoid
Agarose	Bio-Rad
Antibiotics	Sigma
ATP	Sigma
Boric Acid	Merck
Bovine serum albumin	Sigma
Bromophenol blue	Merck
Butan-1-ol	BDH
Calcium Chloride	Merck
Cessium Chloride	Boehrinheim Manheim
Ortophosphate	Merck
Dithiothreitol	Sigma
Ethanol	SARChem
Ethidium bromide	Sigma
Ethylenediaminetetraacetic acid	Protea
Ficoll	Sigma
Hydrochloric acid	BDH
λ DNA	Boehrinheim Manheim
λ H DNA	Boehrinheim Manheim
λ HE DNA	Boehrinheim Manheim
Lysozyme	Boehrinheim Manheim
Magnesium chloride	SMM
Magnesium sulphate	Merck
Nalidixic acid	Sigma
Nick translation kit	Amersham
Nitrocellulose paper	Millipore
Nitrocellulose filters	Millipore
Nutrient broth	Difco
Potassium chloride	SMM
Peptone	Difco
Phenol	BDH

Orthophosphate	Merck
Pronase	Sigma
Propan-2-ol	BDH
Proteinase K	Boehrinheim Manheim
Restriction endonucleases	Boehrinheim Manheim
RNAse	Boehrinheim Manheim
Salmon sperm DNA	Boehrinheim Manheim
Sarkosyl	Sigma
Sodium acetate	Merck
Sodium chloride	Saarchem
Sodium citrate	Merck
Sodium dodecyl sulphate	Merck
Sodium hydrogen phosphate	Merck
Sodium hydroxide	Saarchem
Sucrose	Merck
Trichloroacetic acid	Merck
Tris (hydroxymethyl)methane	BDH
Triptone	Oxoid
XAR-5 X-ray film	Kodak
Yeast extract	Gybco

Chapter 4 RESULTS Page

4 RESULTS

4.1 USE OF THE HYBRID COSMID PCD1253 IN AN ATTEMPT TO DETECT NEW PUTATIVE AGROCIN D286 GENES IN AN EXISTING GENE BANK OF STRAIN D286 WT

The present work started with the intention of finding agrocin D286 genes in a genomic library of strain D286 wt which had been constructed by Botha and Thomson (CSIR, LMBC). For this a ^{32}P labelled probe was made from the 5 kb D286 wt DNA insert in pCD1253, also obtained from Botha and Thomson (CSIR, LMCB), and utilised to probe the genomic library by *in situ* colony hybridisation.

Since the gene bank had been constructed using vector pLAFR1, which is the same vector DNA in pCD1253, the entire pCD1253 DNA could not be used as a probe. Therefore, pCD1253 was digested to completion with EcoRI, the insert DNA separated from pLAFR1 using agarose gel electrophoresis (Figure 4.1) and then isolated by electro elution. The resulting fragment was used to construct the ^{32}P labelled probe.

Probing of the D286 wt gene library failed to single out any new clones carrying DNA sequences that would overlap at least partially with those of the probe. The results of the *in situ* colony hybridization indicate that the probe was effective, as shown by the unequivocal detection of the control colony *E. coli* DHI (pCD1253) (Figure 4.2). Two explanations could be found for the failure of the approach, namely that (i) the genomic library was incomplete or that (ii) the DNA insert in pCD1253 had not originated from strain D286 wt.

Since the average insert size in the gene bank was approximately 20 kb (Botha, personal communication), its 870 members represented the entire cellular DNA of strain D286 wt. However, a number of colonies, repres-

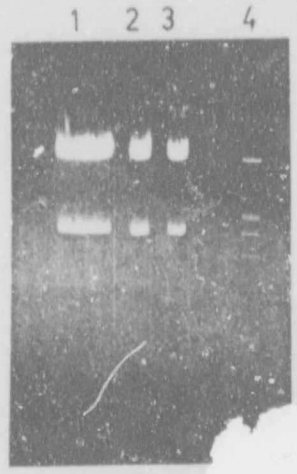

Figure 4.1 Agarose gel electrophoresis of EcoRI total digestion of pCD1253 for the isolation of its putative D286 DNA insert. Lanes 1-3; pCD1253 digested with EcoRI. Lane 4; λ EH (XIII).