JOURNAL OF AGRICULTURAL SCIENCE IN FINLAND
Maataloustieteellinen Aikakauskirja
Vol. 59: 209—215, 1987

Cloning and conjugational transfer of chitinase encoding genes

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Abstract. A genomic library of chromosomal DNA from **Serratia marcescens** was constructed in the broad host range cosmid pLAFR3. Chitinase positive clones were identified on a chitin medium. By conjugational transfer chitinase encoding plasmids were transferred to **Pseudomonas** spp.

Index words: Cosmid cloning, chitinase, biocontrol

Biochemical techniques are available today that make it possible to improve biocontrol agents by genetic engineering. A number of cloning vectors have been developed, which can be used to transform both plant pathogenic and antagonistic bacteria (MACRINA 1984, MILLS 1985, PANOPOULUS and PEET 1985, HOLLOWAY and MORGAN 1986).

Cloning in filamentous fungi has not developed so far as in bacteria. However, several laboratories have developed shuttle vectors able to replicate both in fungi and in *Escherichia coli*. While most attention initially was focused on the yeast *Saccharomyces cerevisiae* and the two filamentous Ascomycetes *Aspergillus nidulans* and *Neurospora crassa,* cloning vectors for antagonistic and plant pathogenic fungi have recently been developed (CULLEN and LEONG 1986, SAUNDERS et al. 1986).

In this paper recent work in the Department of Plant Pathology, University of Wisconsin-Madison will be described to illustrate techniques and methods used in molecular cloning and conjugational transfer of chitinase encoding genes.

Chitin and chitinases

Chitin is a polymer of N-acetylglucoseamine and is a major structural component of the cell walls of fungi with the exception of those in the class of Oomycetes (MONREAL and REESE 1969). It is also found in the exoskeleton of insects, nematodes and other pests, but it is absent in vascular plants and mammals (MUZZARELLI 1977).

The degradation of chitin is catalyzed by chitinases, which hydrolyze chitin to chitodextrins. Chitinases are found in bacteria, (MONREAL and REESE 1969), fungi (ELANGO et

al. 1982), higher plants (PEGG and VASSEY 1973, BOLLER et al. 1983) and animals (MUZZARELLI 1977). Chitinases of the fungus *Beauvaria bassiana* are inducible with D-glucosamine and N-acetylglucoseamin acting as inducers (SMITH and GRULA 1983). Chitin is a high molecular weight oligomer and is unlikely to be the inducer of chitinases. It is possible that a small amount of the enzyme is made constitutively in chitinase producing organisms.

Serratia marcescens is a Gram- negative, entric soil bacterium. It secretes high levels of chitinase, and MONREAL and REESE (1969) found it to be the most active of 100 organisms tested for chitinase production. FUCHS et al. (1986) found *S. marcescens* to produce five different chitinolytic proteins and obtained from a cosmid library clones with a common 9.5 kb *Eco*RI fragment which encoded chitinase activity. Two chitinases were characterized by JONES et al. (1986), and the genes encoding them showed no detectable homology to each other.

Stimulation of chitinolytic organisms have been used successfully in biological control of plant pathogenic fungi. Addition of organic chitin containing amendments to soil have been shown to reduce diseases caused by fungi (PAPAVIZAS and DAVEY 1960, MITCHELL and ALEXANDER 1961, 1962, HENIS et al. 1967, SNEH et al. 1971, SNEH 1981).

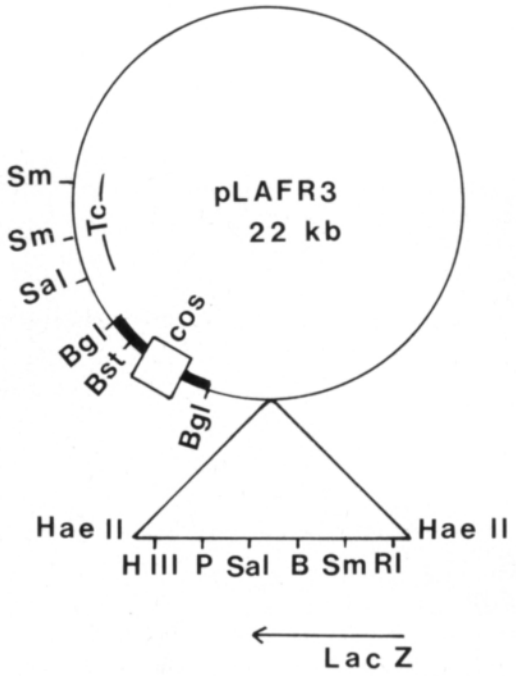

Fig. 1. The broad host range cosmid vector pLAFR3 has single restriction enzyme sites in the LacZ gene.
B = *Bam*HI, HIII = *Hin*dIII, P = *Pst*I, RI = *Eco*RI, Sal = *Sal*I, Sm = *Sma*I.

Cloning of chitinase encoding genes

A genomic library of *S. marcescens* was constructed in *E. coli* HB101 using the cosmid pLAFR3 (FRIEDMAN et al. 1982). The advantages of cosmids are that large DNA-fragments can be cloned *in vitro,* the library can be stored in phage particles, and the DNA is introduced by infection rather than transformation. The cosmid pLAFR3 has a broad host range, contains a gene for tetracycline resistance and has a single *Eco*RI site in a LacZ gene (Fig 1). Following ligation to 15—30 kb foreign DNA it can be packaged *in vitro* into bacteriophage lambda heads.

Chromosomal DNA from *S. marcescens* was isolated and purified by cesium chloride density gradient centrifugation. Digest with *Eco*RI and separation of the fragments on a sucrose gradient was carried out using standard techniques (MANIATIS et al. 1982). Fractions containing 15 to 30 kb fragments were pooled and used for ligation (Fig. 2).

The cosmid vector was cut with *Eco*RI and dephosphorylated using Bacterial Alkaline Phosphatase purchased from International Biotechnologies Inc., New Haven, CT., to avoid self-ligation of the vector. Ligation of the *S. marcescens* DNA and the cut vector was carried out at 14°C for 24 hrs. Recombinant cosmids were packaged into lambda heads using »Packagene» purchased from Promega Biotech., Madison, WI, and transfected into *E. coli* HB101. Following overnight growth on tetracycline containing plates, transformants were transferred to master

plates and replicated onto CA overlay plates for chitinase assay.

Chitin was purified from a commercial preparation (Sigma, St. Luis, MO), by the method used by VESSEY and PEGG (1973). Assay plates were prepared by adding 10 ml chitin agar (CA) on top of 15 ml mineral medium containing 5 mM glucose. Chitinase activity was detected within 2—3 days at 37°C as a zone of clearing around chitinase producing colonies. Chitinase positive clones were isolated from a genomic library.

Plasmids from chitinase active clones were analyzed using the miniprep protocol of MANIATIS et al. (1982). The clone with plasmid pLES8 is representative of clones with an 18 kb insert in the vector plasmid (Fig. 3).

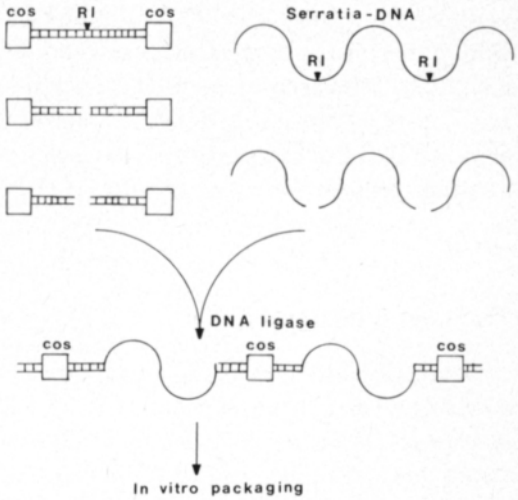

Fig. 2. Schematic presentation of cosmid cloning and *in vitro* packaging.

Subcloning

Chitinase positive clones were grown in LB medium with tetracycline, and plasmids were isolated according to the alkaline lysis method (MANIATIS et al. 1982). To obtain clones with single band *Eco*RI inserts in pLAFR3, plasmids were cut with *Eco*RI, ligated, and competent *E. coli* TB1 cells were transformed. The transformants were plated on LB medium containing X-gal. After incubation overnight transformants with inserts in the *Eco*RI site in the Lac Z gene could be selected as white colonies. Transformants were replicated onto chitin medium. Plasmids from chitinase positive clones were analyzed by the miniprep protocol. Subclone pLES81 was isolated as representative of the subclones which contained a single 18 kb *Eco*RI fragment in the vector, while subclone pLES51 represented subclones with a single 9.4 kb *Eco*RI insert.

Subcloning into the smaller, high copy number plasmid pBR325 was done to facilitate mapping of the DNA fragments. The plasmid has an *Eco*RI site in the gene for chloramphenicol resistance. Following *Eco*RI digest of the plasmids pLES81 and pBR325, ligation and transformation of HB101 cells, transformants were plated on plates containing ampicillin and tetracycline. By replica

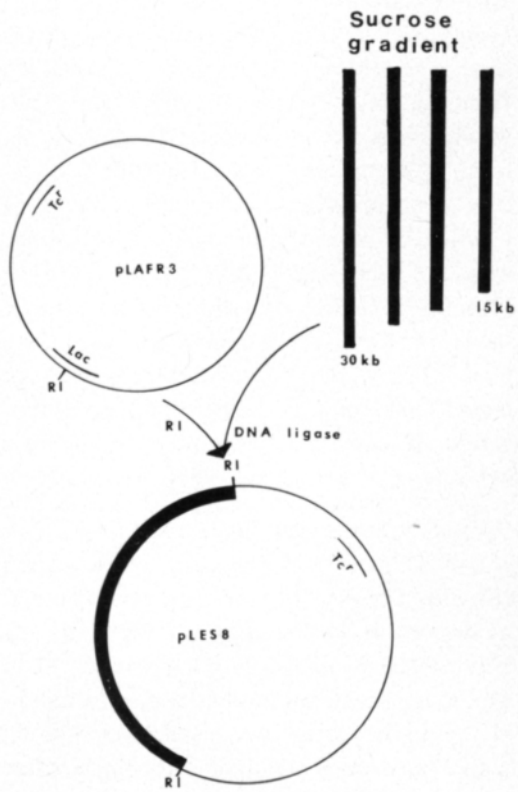

Fig. 3. *Eco*RI fragments of chromosomal DNA from *Serratia marcescens* were separated by ultracentrifugation in sucrose gradients. Fractions with fragments in the 15 to 30 kb size range were used for ligation to *Eco*RI cut pLAFR3.

plating onto plates with all three antibiotica, chloramphenicol sensitive clones could be identified. They were then tested for chitinase activity as previously described, and the chitinase active subclone pLES83 was isolated as a representative subclone in pBR325 (Fig. 4).

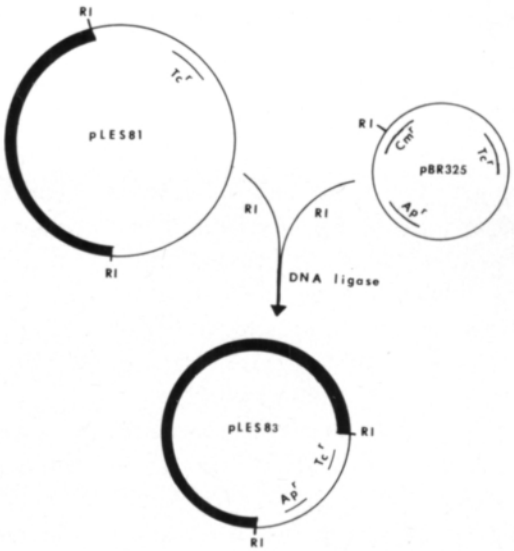

Fig. 4. Subcloning into the high copy number plasmid pBR325.

Transposon mutagenesis

Generation of mutations by transposons is a useful tool in mapping of genes. A Tn3 LacZ transposon made by STACHEL et al. (1985) serves both as a transposon mutagen and generates gene fusions that can be exploited to study gene expression. In gene fusion the control sequence of the gene under study is placed in front of the coding sequences of a reporter gene whose product can be assayed. By measuring the reporter gene product, genetic and environmental factors that affect the gene expression can be determined (SILHAVY and BECKWITH 1985). The Tn3-HoHo1 transposon generates random mutations in bacterial plasmids, and the production of beta-galactosidase, the LacZ gene product, is placed under the control of the gene into which Tn3-HoHo1 has inserted. The plasmid pHoHo1 carrying the transposon lacks transposase and depends on the helper plasmid pSSe, which provides transposase in trans. Thus, once the transposon has been inserted, it cannot selftranspose in the new genome (STACHEL et al. 1985).

First the strain carrying the Tn3 transposon is transformed by the target plasmid pLES8 (Fig. 5). Then the transformed cells are used as donors in a triparental mating using the helper plasmid pRK2013 (DITTA et al. 1980). The recipient strain has resistance to nalidixic acid. By plating the transconjugants on media containing nalidixic acid, tetracycline and ampicillin, only cells with the transposon in the target plasmid could grow. Tetracycline resistance is encoded by the target plasmid and the transposon carries a gene for ampicillin resistance (Fig. 6).

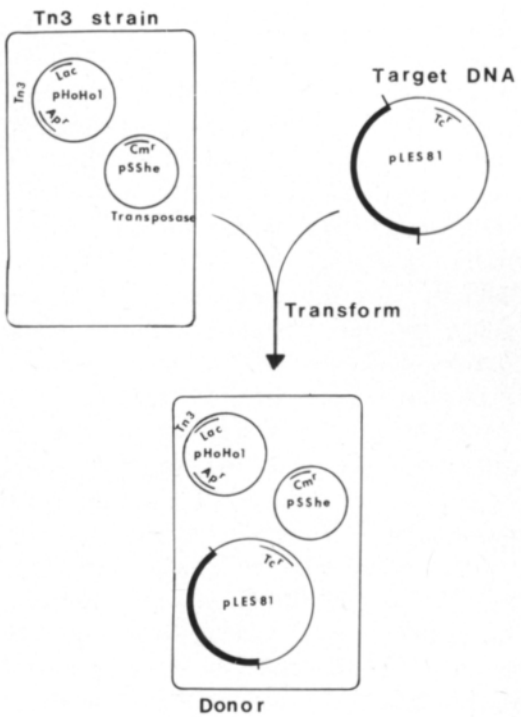

Fig. 5. First step in transposon mutagenesis. The target plasmid DNA is transformed into a strain containing the Tn3 carrying plasmid pHoHo1 and the transposase encoding plasmid pSShe.

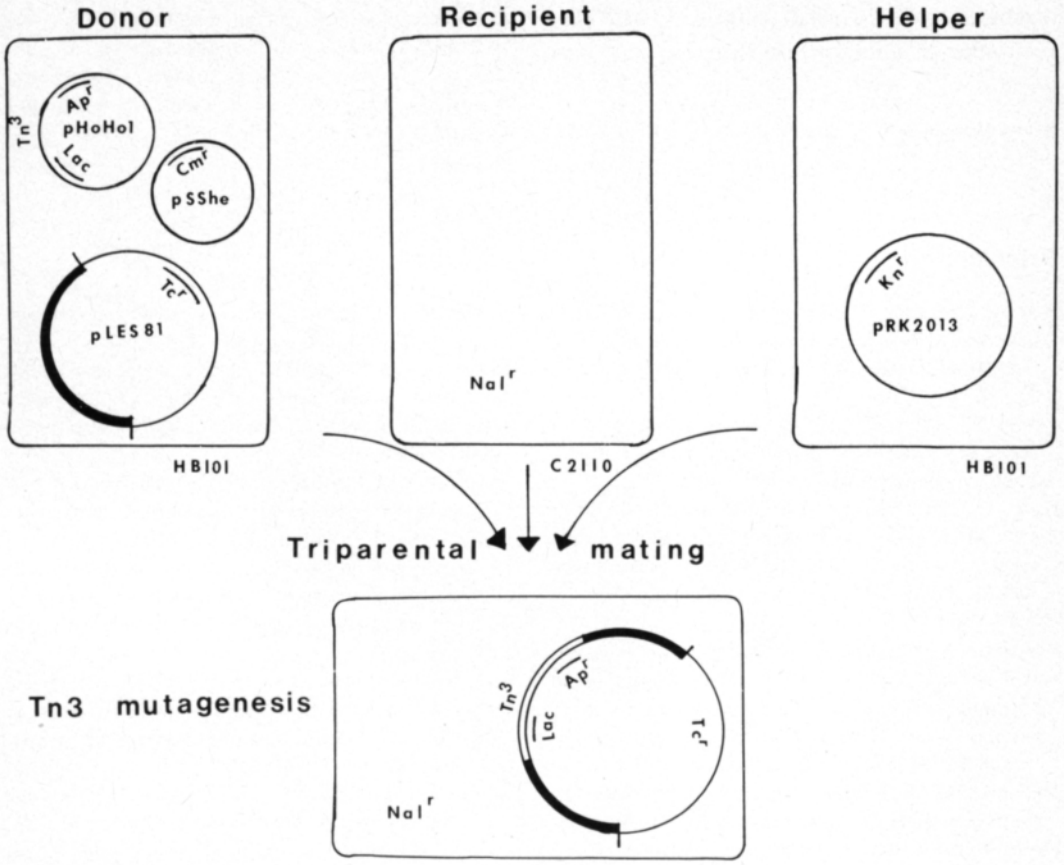

Fig. 6. Transformed cells are used as donors in a triparental mating. Only cells with Tn3 transposon in the target plasmid will be able to grow on a selective medium containing nalidixic acid, tetracycline and ampicillin.

Transconjugants from transposon mutagenesis experiments were tested for chitinase activity on chitin medium. A number of mutants with loss of chitinolytic activity and Tn3 insertions have been found and work is in progress to map the sites of insertion.

Conjugational transfer of the chitinase genes to *Pseudomonas* spp.

To exploit the chitinolytic activity in biocontrol of plant pathogenic fungi the coding sequences of the chitinase gene have to be transferred into plant colonizing bacteria. In a triparental mating the helper plasmid pRK2013 was used. The plasmids pLES51 and pLES81 with 9.4 and 18 kb *Eco*RI fragment inserts respectively, were transferred into fluorescent *Pseudomonas* strains and chitinase positive transconjugants were selected. The biocontrol potential of chitinase positive transconjugants is currently being studied.

Conclusion

Modern molecular biology techniques offer powerful tools for analyzing the interactions between organisms. With our increasing understanding of the biological phenomena we may be able to improve the efficiency of biocontrol agents by transferring useful genes between microorganisms. With bacteria that kind of technology is available today. A number of laboratories are making intense efforts to develop fungal cloning vectors which un-

doubtedly will be used to improve the performance of antagonistic fungi.

Acknowledgements. I gratefully thank Dr. A. H. Ellingboe, Department of Plant Pathology, University of Wisconsin-Madison, who introduced me to molecular cloning and gave me excellent conditions for research during my sabbatical year. I also thank The Agricultural Research Council of Norway for financial support.

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Ms received August 19, 1987

Kitinaasia koodaavien geenien kloonaus ja konjugaatio

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Geeniteknologian menetelmät ovat jo kehittyneet niin pitkälle, että niillä voidaan parantaa kasvitautien biologisessa torjunnassa käytettävien antagonistimikrobien ominaisuuksia. Yhdysvalloissa Madisonin yliopiston kasvipatologian laitoksella on siirretty kitinaasientsyymiä koodaavia geenejä *Pseudomonas*-bakteeriin parantamaan sen sienitautien torjuntatehoa.

Monien sienipatogeenien soluseinässä on kitiini-nimistä ainetta, joka hajoaa kitinaasientsyymin vaikutuksesta. Eräät bakteerit, esimerkiksi *Serratia marcescens*, erittävät runsaasti kitinaasia. Kitiiniä hajottavia organismeja on onnistuneesti käytetty biologisessa torjunnassa.

Serratia marcescens -bakteerin kromosomaalisesta DNA:sta tehtiin genomikirjasto laajakirjoisen pLAFR3 -kosmidivektorin avulla. Kirjastosta poimittiin kitinaasia koodaavat geenit eristämällä kitinaasia tuottavat kloonit erikoisselektioalustalla. Tämän jälkeen kitinaasipositiiviset kloonit subkloonattiin ja niitä mutagenisoitiin Tn^3-transposonilla DNA-fragmenttien paikantamisen helpottamiseksi. Kitinaasia koodaavat geenit konjugoitiin antagonistiseen *Pseudomonas fluorescens* -bakteeriin, jonka tehoa sienitautien biologisessa torjunnassa tutkitaan parhaillaan.