Acta Biotechnol. 14 (1994) 2, 119–129 Akademie Verlag

# Expression of the 2,4-D Degrading Plasmid pJP4 of *Alcaligenes eutrophus* in *Rhizobium trifolii*

FENG, L.[1], ROLFE, B. G.[2], GARTNER, E.[2], VAN ZWIETEN, L.[1], KENNEDY, I. R.[1] *

[1] University of Sydney
Department of Agricultural Chemistry and Soil Science
Ross Street Building A03
Sydney, N. S.W. 2006, Australia
[2] Australian National University
Research School of Biological Sciences
Plant Microbe Interaction Group
Canberra A.C.T. 2601, Australia

## Summary

The 2,4-dichlorophenoxy acetic acid (2,4-D) degrading plasmid, pJP4, was transferred into *Rhizobium trifolii* ANU843 from its nature host *Alcaligenes eutrophus* JMP134 by conjugation. The ability to degrade 2,4-D was expressed in the transconjugant ANU843p as shown by a total loss of UV-absorbent compounds and by gas chromatographic analysis. However, the transconjugant was unable to grow on 2,4-D alone. When the transconjugant strain ANU843p was inoculated onto white and subterranean clover plants in laboratory trials, the transconjugant retained the capacity of nodulation, but the nitrogen-fixation activity was diminished, particularly in the case of subterranean clover. The plasmid in the transconjugant was stable in nodules for at least nine weeks after inoculation and could be of value in applications requiring the protection or removal of the 2,4-D involving cometabolism with plant substrates.

## Introduction

Halogenated aromatic compounds have been produced industrially on a large scale and used mainly in agriculture as pesticides for several decades. Despite their economic benefits to agriculture, there is no doubt that using such compounds, through the contamination and persistence of residues in soil and water, presents serious environmental problems. The possibility of microbial degradation of these compounds is an important consideration for their elimination [1–3].

Recently, several genera of soil bacteria have been found to degrade and utilize some of those compounds [1–3]. 2,4-Dichlorophenoxyacetic acid (2,4-D) is especially known to be degraded by a number of different bacteria, such as *Acinetobater*, *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Corynebacterium*, *Flavobacterium*, *Pseudomonas*, and *Xanthobacter*, and the related genes are mostly plasmid borne [1, 4–12]. Among those 2,4-D degrading bacteria, *Alcaligenes eutrophus* JMP134 is one of the best characterized organisms, con-

* Corresponding author

taining a plasmid encoding the genes responsible for 2,4-D degradation [8, 13, 14]. The plasmid, which is very well characterized genetically [8, 15–19], designated pJP4, is about 80 kilobase (kb) in size, and belongs to the IncP1 incompatibility group. It is transferable by conjugation to the strains of *Escherichia coli*, *Rhodopseudomonas sphaeroides*, *Rhizobium* sp., *Agrobacterium tumefaciens*, *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Acinetobacter calcoaceticus*, also conferring resistance to $HgCl_2$ and degradation of 2,4-D [8, 20].

In this study, attempts were made to transfer the plasmid pJP4 from *Alcaligenes eutrophus* JMP134 into *Rhizobium trifolii* ANU843 and *Rhizobium* sp. ANU240.

Rhizobia are able to grow in the rhizosphere of leguminous plants, also nodulating them, producing a nitrogen-fixing symbiosis with those plants. The construction of this transconjugant of *Rhizobium trifolii* potentially enables the degradation of 2,4-D in the plant rhizosphere and could provide a demonstration of a model for the protection of plants from herbicides and pesticides.

## Materials and Methods

### *Bacterial Strains*

*Alcaligenes eutrophus* JMP134 containing the plasmid pJP4 was obtained from Danny LLEWELLYN of CSIRO, Division of Plant Industry, Canberra. *Rhizobium trifolii* ANU843, *Rhizobium* sp. ANU240 and *E. coli* HB101 were provided by B. ROLFE of the Research School of Biological Sciences, Australian National University. The transconjugants *R. trifolii* ANU843p, *Rhizobium* sp. ANU240p and *E. coli* HB101p were obtained in this study.

### *Media and Growth Conditions*

A TY medium containing 5 g tryptone, 3 g yeast and 0.9 g/l $CaCl_2$ was used for the growth of all the bacteria. A TMR medium [21] containing 0.5 g/l $(NH_4)_2SO_4$, 10 g/l sucrose and other elements was used for the selection and growth of the *Rhizobium* strains. TMR without a carbon source was used as a wash for the cell suspension and the medium for the resting-cell assay of the 2,4-D degradation when amended with 2,4-D as a sole carbon source. A BMM medium [22] containing 0.5 g/l Na glutamate, 0.5 g/l yeast, 3 g/l mannitol, and other elements was used for the germination of white and subterranean clover seeds. A BMM mannitol medium [22], the same as the BMM medium except for the content of 36 g/l mannitol, was used for the reisolation of bacteria from plant nodules. A modified FÄHRAEUS medium [23] was used for growing white and subterranean clover seedlings in nodulation and nitrogen fixation trials. The concentration of $HgCl_2$ used for the selection was 40 µg/ml for *A. eutrophus* JMP134 and *E. coli* HB101p and 10 µg/ml for *Rhizobium* strains. The concentration of streptomycin was 300 µg/ml. 2,4-D was prepared as 20 mg/ml of stock in 0.1M $NaH_2PO_4$ [4] and was filter-sterilized before being used. The level of 2,4-D used in the media ranged from 0.2 to 4.6 mM. All the cultures were grown at 29 °C. Liquid cultures were incubated on an orbital shaker at 200 rpm on a BRAUN Certomat R shaker. Solid media contained 1.5% agar (SIGMA).

### *Conjugation*

Donor and recipient strains were grown overnight prior to the mixing on TY agar plates. Controls were performed with the donor and recipient alone. The crosses of the donor and recipient were grown overnight and replicated on selective media. These were: TMR with 10 µg/ml $HgCl_2$ for *R. trifolii* ANU843p; TMR with 300 µg/ml streptomycin plus 10 µg/ml $HgCl_2$ for *Rhizobium* sp. ANU240p and LB with 300 µg/ml streptomycin plus 40 µg/ml $HgCl_2$ for *E. coli* HB101p. The transconjugants were purified on selective medium respectively by several subcultures.

### *Plasmid Visualization*

Plasmids were visualized by a rapid screening technique according to PLAZINSKI *et al.* [24], except for 200 µl of 0.2% sarkosyl, 80 µl of lysis mixture and 10 µl proteinase K were used. TY medium was used for the preparation of cultures.

### *Growth and Degradation of 2,4-D*

Each strain was grown in 10 ml TMR for 2 days and a portion was used to inoculate the shake flasks containing 50 ml of fresh TMR medium which contained sucrose supplied with 0.2 mM of 2,4-D to an initial $A_{600}$ of 0.05. The cultures were incubated while being shaken at 29 °C. The growth of cells was monitored by measuring $A_{600}$ against a blank of TMR medium. The degradation of 2,4-D was detected by measuring the loss of UV absorbent compounds [4] as follows: 1 ml of culture sample was centrifuged at 12,000 rpm for 5 min using a BECKMAN Microfuge E, and the supernatant was then used to measure $A_{283}$ at which 2,4-D has a maximum absorbance in a BECKMAN DU64 spectrophotometer.

### *Resting-Cell Assay*

The degradation of 2,4-D by the resting cells of *R. trifolii* strains was determined as follows: each strain was grown in 100 ml of TMR to an $A_{600}$ of 0.8 – 1.0. Cells were harvested by centrifugation and washed twice using TMR without a carbon source. Then the cells were resuspended in 100 ml TMR without any other carbon sources, amended with 2,4-D and divided into two flasks. The concentration of 2,4-D used ranged from 0.2 to 4.6 mM. A 1 ml sample was taken at each time point, and the degradation of 2,4-D was assayed by measuring the UV absorbance ($A_{283}$) and by GC analysis.

### *Gas Chromatographic Analysis of 2,4-D*

Culture samples from the resting-cell assay were used. A 2 ml sample of culture was placed in a liquid/liquid extractor (Mixxor®) and 10 ml of 25% acetone in hexane (Nanograd®) with 10 drops of concentrated sulphuric acid were added. Following 50 strokes of the piston, the organic phase was removed and the procedure was repeated. The organic phases were combined and dried by filtering through 2 g of anhydrous sodium sulphate. The filter was further rinsed with 5 ml of hexane and combined with the sample in a 50 ml pear-shaped flask. The organic phase was evaporated to 0.5 ml by rotary evaporation and the 2,4-D was derivatized to 2,4-D methyl ester using the following procedure: 2 ml of 0.5% KOH and 2 ml of 14% of the $BF_3$/methanol complex were added and refluxed with a water jacketed condenser for 20 min. After cooling, 5 ml of saturated aqueous NaCl was added through the condenser and the 2,4-D methyl ester was extracted by shaking with 10 ml of hexane. Hexane was removed, and the sample was dried with anhydrous sodium sulphate and diluted as required. All the samples were stored in vials in a freezer prior to the analysis.

The 2,4-D methyl ester concentration was determined by gas-chromatography. A HEWLETT-PACKARD 5890 Series 2 Gas Chromatography fitted with a $Ni^{63}$ Electron Capture Detector was used. 1 µl of sample was injected by a model 5971 autosampler. A solvent concentration method with a temperature programme was used. The column was a 30 m by 0.32 mm DB17 capillary column (J & W). The injector temperature was 210 °C, and the detector temperature was 300 °C. External 2,4-D methyl ester standards were used, and the chromatograms were integrated by HEWLETT-PACKARD software on a personal computer and stored on disk.

### *Plant Growth*

White and subterranean clover seeds were surface-sterilized for 15 min with 6% (v/v) sodium hypochlorite and germinated on BMM agar plates as described [22]. The seedlings were transferred onto FÄHRAEUS plates [23], and the bacteria were inoculated using the rapid plate-screening method [22]. The inoculated plates were incubated vertically with a temperature of 27 °C for 18 hours a day and 19 °C for 6 hours a night. The plants were assayed after 3 to 4 weeks with reference of the control plants for the tests of the ability of nodulation, $N_2$ fixation and stability of the plasmid pJP4.

### *Stability of the Plasmid pJP4 for Rhizobium*

Bacteria were reisolated from nodules as described by ROLFE *et al*. [22]. The diluted cell suspension from nodules was spread on BMM mannitol agar. A single colony from the BMM mannitol agar was used to inoculate 5 ml of TMR medium supplied with 0.2 mM 2,4-D for the 2,4-D degradation test by measuring $A_{283}$. The degradation of 2,4-D indicated the presence of the plasmid pJP4.

*Assay for the Nitrogen Fixation*

The nitrogenase activity was assayed by the acetylene reduction test, using 10% of $C_2H_2$ in 28 ml McCartney bottles containing one plant. The ethylene production was measured using a SHIMADZU GC8A gas chromatograph; 0.5 ml of gas sample was injected into a 1 m column filled with Porapak T (WATERS MILLIPORE) with an injection temperature of 140 °C and a column temperature of 130 °C. The $C_2H_4$ production was calculated by the peak area in comparison with 0.5 ml of standard of 1/10000 $C_2H_4$ in air.

## Results

*Transfer of the Plasmid pJP4 from A. eutrophus JMP134 into R. trifolii ANU843, Rhizobium* sp. *ANU240 and E. coli HB101*

The plasmid pJP4 was transferred into *R. trifolii* ANU843 and *Rhizobium* sp. ANU240, as well as *E. coli* HB101 which was used as a control strain from its natural host *A. eutrophus* JMP134 by conjugation. The purification of the transconjugants was carefully performed on the selective media and no contaminations were observed. Electrophoresis results show that each transconjugant has an extra band compared to its parent strains, and the extra band is at the same position as *A. eutrophus* JMP134 (Fig. 1).

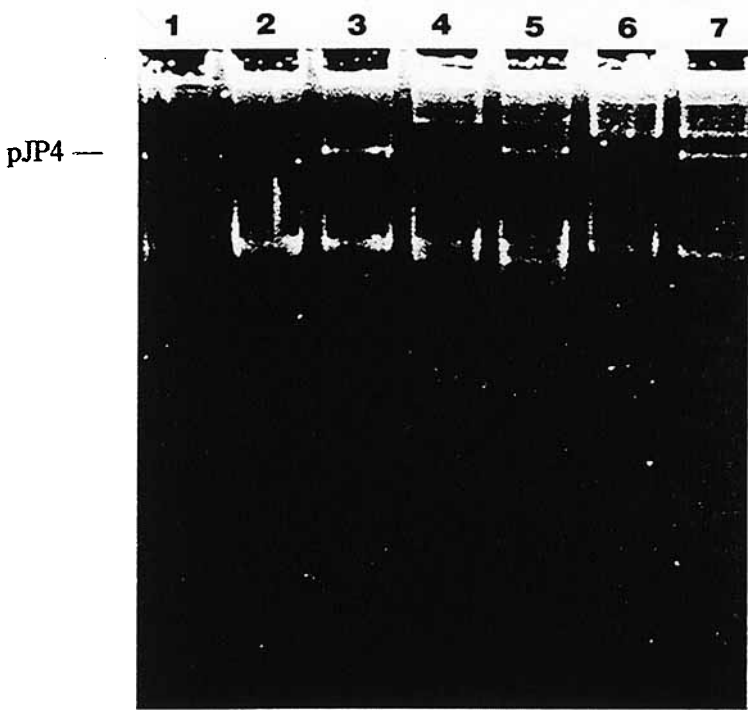


Fig. 1. Plasmid gel profile
Lane 1, *A. eutrophus* JMP134 (the parent strain of plasmid pJP4)
Lane 2, *E. coli* HB101
Lane 3, *E. coli* HB101p (pJP4)
Lane 4, *Rhizobium* sp. ANU240
Lane 5, *Rhizobium* sp. ANU240p (pJP4)
Lane 6, *R. trifolii* ANU843
Lane 7, *R. trifolii* ANU843p (pJP4)

*Expression of the Resistance to $HgCl_2$*

The resistance to $HgCl_2$ was expressed in all the recipients. The degree of resistance differed with each strain. Growth was obtained with 10 μg/ml $HgCl_2$ for *R. trifolii* ANU843p and *Rhizobium* sp. ANU240p, in contrast to the absence of growth in the parent *Rhizobium* strains with 5 μg/ml, to 40 μg/ml for *A. eutrophus* JMP134 and *E. coli* HB101p.

*Growth and Degradation of 2,4-D*

The ability of the transconjugant strain ANU843p to degrade 2,4-D when grown in TMR (containing sucrose) amended with 2,4-D (0.2 mM) is shown in Fig. 2. No degradation was detected at the end of day one by measuring a loss of UV-absorbent compounds at 283 nm ($A_{283}$), while the growth was just started as indicated by $A_{600}$. At the end of day two, the growth was in the middle of the log-phase and about a 20% decrease of $A_{283}$ was detected. At the end of day three, a major decrease of $A_{283}$ (about 80%) was detected, and the growth reached its late log-phase or early stationary phase. No further decrease of $A_{283}$ was detected after day three. A decrease of $A_{283}$ was not detected for the parent strain ANU843, which had the same growth pattern as the transconjugant during the whole period of incubation.

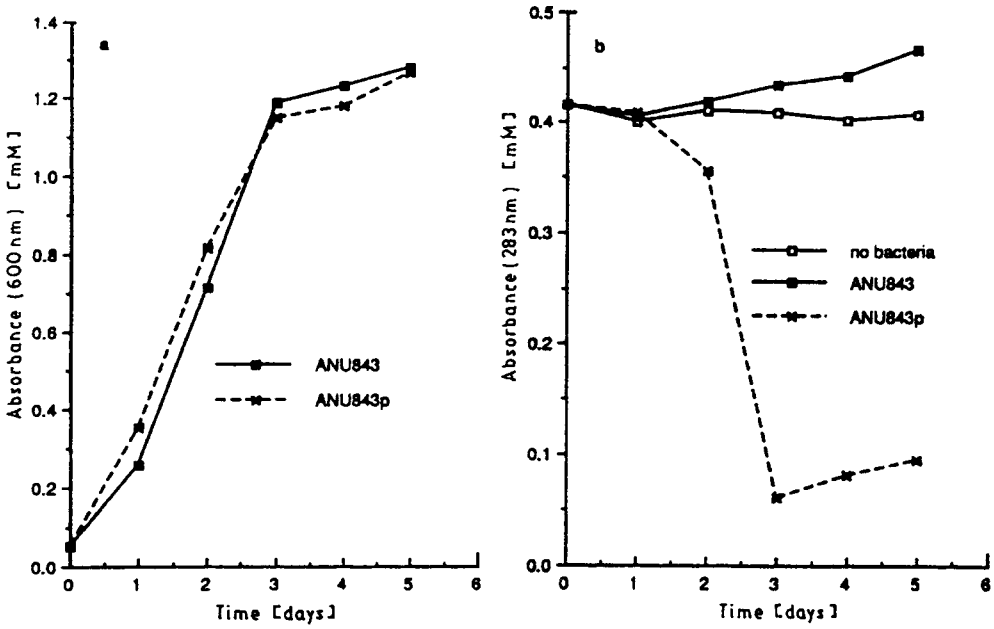


Fig. 2. Growth and degradation of 2,4-D in the presence of sucrose by ANU843p
A TMR medium (containing 10 g/l sucrose) amended with 2,4-D (0.2 mM) was used. The initial cell density at $A_{600}$ was 0.05. The growth was monitored by the optical density at 600 nm (a), and the 2,4-D degradation was monitored by measuring the loss of total UV-absorbent compounds at 283 nm (b). All the data are means of duplicates.

*Degradation of 2,4-D by the Resting Cells of the Strain ANU843p*

The ability to degrade 2,4-D (0.2 mM) by the resting cells of the transconjugant strain ANU843p is shown in Fig. 3. Samples from different periods of incubation were assayed

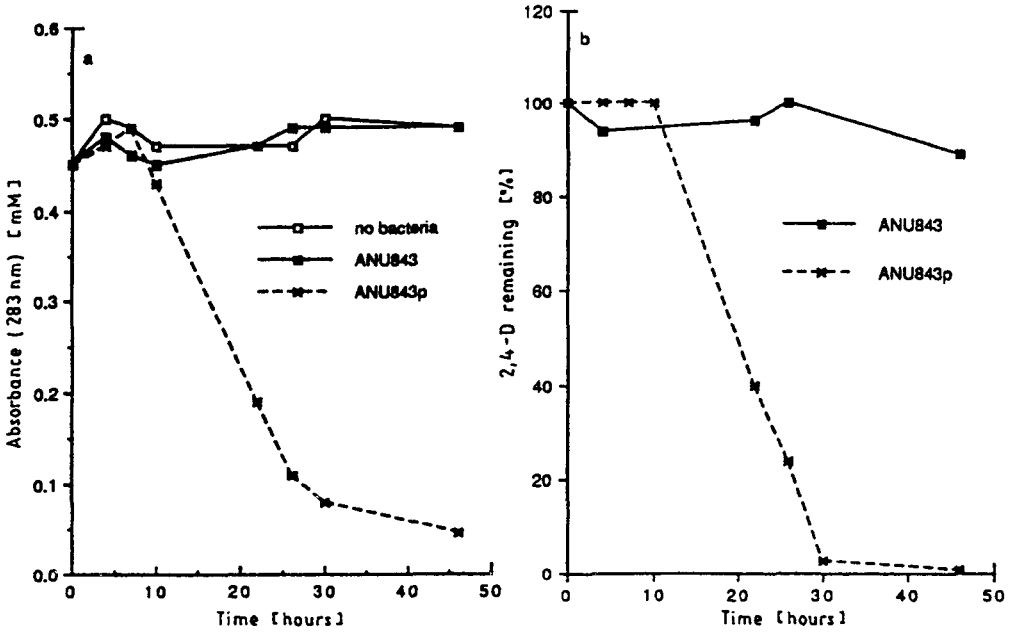


Fig. 3. Resting cell assay for the 2,4-D degradation by ANU843p
The cells were washed twice with TMR without sucrose before they were resuspended into TMR without sucrose amended with 2,4-D (0.2 mM). The initial cell density at $A_{600}$ was 0.9. The degradation of 2,4-D was monitored by measuring the loss of total-UV absorbent compounds at 283 nm (a) and gas chromatography (b). All the data are the means of duplicates.

for the disappearance of 2,4-D by measuring the loss of total UV-absorbent compounds at 283 nm (3a) and by gas chromatography (3b). A decrease of $A_{283}$ was not detected in the first 10 hours with the transconjugant strain ANU843p. About a 60% decrease of $A_{283}$ was detected after 22 hours and another 80% loss of $A_{283}$ after 26 hours. The GC analysis showed the same degradation pattern without a disappearance of 2,4-D in the first 10 hours, a 60% disappearance after 22 hours, a 80% one after 26 hours, and a 95% one after 30 hours. With either method, no degradation of 2,4-D (0.2 mM) was detected using the parent strain ANU843.

*Effect of the Initial 2,4-D Concentration on the Degradation by Resting Cells of ANU843p*

The effect of the initial 2,4-D concentration on the degradation of 2,4-D by the resting cells of the transconjugant strain ANU843p was investigated, and the result is shown in Fig. 4. Washed cells were resuspended into TMR in the absence of sucrose amended with different levels of 2,4-D (0.2, 0.7, 1.7, and 4.6 mM, respectively) with a constant starting $A_{600}$ of 0.8. Samples were taken during the incubation to assay for the degradation of 2,4-D by measuring a total loss of UV-absorbent compounds ($A_{283}$). At an initial 2,4-D level of 0.2 mM, no decrease of $A_{283}$ was detected for the first eight hours of incubation, about a 30 percent decrease of $A_{283}$ at the end of day one, an 80 percent decrease was detected at the end of day two, and no further decrease of $A_{283}$ was detected after day three using the transconjugant strain ANU843p. At an initial 2,4-D level of 0.7 mM, no decrease of $A_{283}$ was detected at the end of day one. There was about a 35 percent decrease of $A_{283}$ at the end of day two, a 64 percent decrease at the end of day three, an 82 percent decrease at the end of day four, and no more decrease of $A_{283}$ was detected after day five.

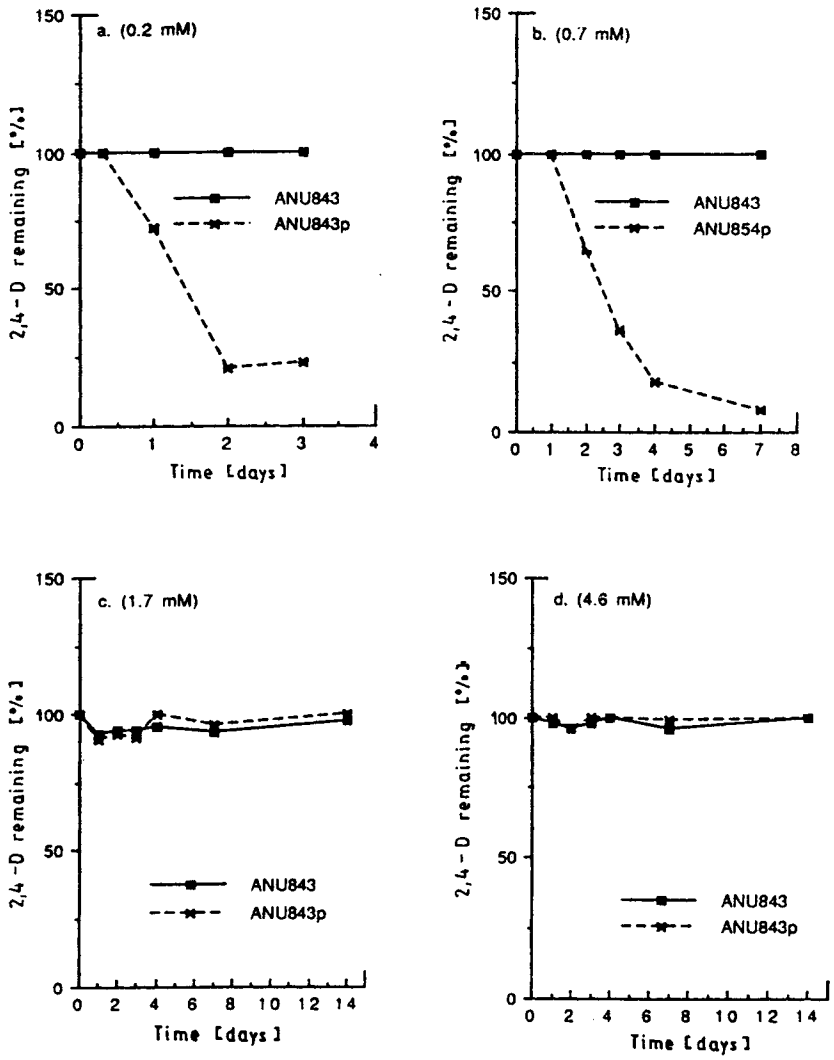


Fig. 4. Effect of the initial 2,4-D concentration on the degradation of 2,4-D by resting cells of the transconjugant strain ANU843p

At an initial 2,4-D level of 1.7 mM and 4.6 mM, a decrease of $A_{283}$ could not be detected with the transconjugant strain ANU843p during a 14-day incubation period. There was no decrease of $A_{283}$ by using the parent strain *R. trifolii* ANU843 in any of these cases.

*Plant Assay*

When grown on agar plates in a growth cabinet, the transconjugant *R. trifolii* ANU843p retained the capacity to nodulate white and subterranean clovers. There were no significant differences in the number or size of nodules between the parental strain ANU843 and the transconjugant ANU843p for white clover. Smaller nodules and greater nodule numbers were observed for the transconjugant ANU843p when applied on subterranean clover in

comparison to the parental strain ANU843, with a noticeable lack of the leghaemoglobin pigment. For nitrogenase activity assays, with the transconjugant ANU843p, there was a slightly less $C_2H_2$ reduction activity in white clover and much less in subterranean clover, in comparison to the parental strain ANU843 (Tab. 1). The results showed the large reduction of the $N_2$ fixing ability for the transconjugant ANU843p in subterranean clover, but the activity was largely maintained for ANU843p in white clover.

Tab. 1. Nodule formation and $C_2H_2$ reduction activity in white and subterranean clovers 4 weeks after inoculation with ANU843 and ANU843p

| Plant/Strain | Nodule number[1] [per plant] | Acetylene reduction[2] [nmol $C_2H_4$/plant/h] |
|---|---|---|
| White clover/ANU843 | 8 | 50.6 |
| White clover/ANU843p | 9.8 | 38.7 |
| Subterranean/ANU843 | 12.2 | 61.5 |
| Subterranean/ANU843p | 17.5 | 15.6 |

1 – the mean value of 31 to 36 plants
2 – the mean value of 7 to 11 plants

*Stability of the Plasmid pJP4 in the Transconjugant ANU843p*

The stability of the plasmid pJP4 in the transconjugant strain ANU843p was monitored by assaying ANU843p reisolated from nodules for the ability to degrade 2,4-D. A single clone of the transconjugant ANU843p reisolated from nodules was subcultured and used to inoculate next 'generation' plants after confirming the presence of the plasmid pJP4. For each generation, fifty colonies reisolated from nodules of white or subterranean clover were tested for the degradation of 2,4-D. All the 50 samples had a major decrease of the UV absorbance at 283 nm within two days and both controls remained unchanged (data not shown). Our results showed that the plasmid pJP4 is stable in the transconjugant ANU843p for at least three 'generations' (nine weeks) with both white and subterranean clovers.

## Discussion and Conclusion

*Transfer of the Plasmid pJP4*

The plasmid pJP4 has been characterized as a broad-host range plasmid of the incompatibility group P1 [8]. Thus, it was not unexpected that the transfers of the plasmid from its natural host, *A. eutrophus* JMP134, into the *R. trifolii* strain ANU843, *Rhizobium* sp. ANU240 and *E. coli* HB101 (as a control strain) were successful. *E. coli* strains were already reported to be a recipient of the plasmid pJP4 [8, 18, 20]. The gel electrophoresis result showed that an extra band corresponding to the plasmid pJP4 appeared in all the recipients, and the extra band was at the same position as its donor *A. eutrophus* JMP134, suggesting that the plasmid was transferred entirely without major deletion (Fig. 1).

*Expression of the Resistance to $HgCl_2$*

The resistance to mercuric chloride was expressed in all the recipients, but the degree of the resistance was different from each recipients. FRIEDRICH *et al.* [20] have reported that the degree of the $HgCl_2$ resistance was dependent on the degree of the sensitivity of the respective parental strains. The transconjugant of *E. coli* HB101 had the same level of resistance as its donor *A. eutrophus* JMP134 at 40 μg/ml as reported before [8]. *R. trifolii* ANU843p and *Rhizobium* sp. ANU240p were resistant to $HgCl_2$ only up to 10 μg/ml.

*Expression of the 2,4-D Degrading Ability in ANU843p*

Our studies clearly showed that the 2,4-D degrading ability was expressed in the transconjugant ANU843p as proven by either a decrease of the UV absorbance or GC analysis (Figs. 2 and 3). There was a lag phase of 24 hours when growing on sucrose and 2,4-D (Fig. 2) and 10 hours for resting cells (Fig. 3), before the degradation of 2,4-D was started. The lag phase period was probably due to the first time exposure of the ANU843p culture to 2,4-D, and a small initial biomass concentration at that time is required to increase the cell population to a level where a significant degradation is possible. SINTON *et al.* [25] suggested that an exposure of cultures to 2,4-D for the first time or a small initial biomass concentration results in a lag phase before the degradation.

When the 2,4-D degradation was monitored by measuring the loss of total UV absorbent compounds, it was impossible to indicate a 100% decrease of $A_{283}$. This was probably due to the presence of small amounts of UV-absorbent compounds other than 2,4-D such as DNA from dissolved dead cells. Gas chromatographic analysis confirmed the complete degradation of 2,4-D by ANU843p.

A second carbon source (sucrose was used in this study) as well as 2,4-D was needed to ensure the degradation of 2,4-D by ANU843p if resting cells of ANU843p were not used. No evidence was obtained that ANU843p can grow on and degrade 2,4-D as the sole carbon source (using 0.2 to 4.6 mM), either in a liquid medium or on agar plates in our study. SHORT *et al.* [26] showed that the 2,4-D degradation by pJP4 was expressed in a transconjugant of a *Pseudomonas* strain without supporting the cell growth as a sole carbon source. In our study, co-metabolism is also suggested for the degradation of 2,4-D with ANU843p.

It has been shown that the plasmid pJP4 encodes enzymes to convert 2,4-D to 2-chloromaleylactate [14, 15]. Additional enzymes including maleylacetate reductase converting 2-chloromaleylactate into 3-oxoadipate were required for the further metabolism of 2-chloromaleylacetate, providing the TCA cycle intermediates are not plasmid pJP4 borne and the genes responsible for those enzymes apparently are located on chromosomal DNA in *A. eutrophus* JMP134 [15]. KUKOR *et al.* [27] showed that a transconjugant of the *Pseudomonas* strain carrying the plasmid pJP4 gained the ability to grow on 2,4-D as a sole carbon source when maleylacetate reductase was provided from another source. In our study, the most likely reason for ANU843p failing to grow on 2,4-D as a sole carbon source is the lack of maleylacetate reductase or other enzymes for the further metabolism of the pJP4 degradative pathway product. As reported before [8, 20], within a wide range of transconjugants of the plasmid pJP4, the ability to degrade 2,4-D is only expressed in *A. eutrophus*, *A. paradoxus* and *P. putida*. The expression of the 2,4-D degradation ability acting as cometabolism is significant in the transconjugant *R. trifolii* ANU843p. It suggests that the ability to degrade 2,4-D could be expressed in more transconjugants from different genera.

*Toxicity of 2,4-D to the Tansconjugant ANU843p*

A high concentration of substrates often results in an inhibition of the degradation, due to the toxicity of these compounds [25]. DITZELMÜLLER *et al.* [7] have reported on the inhibition of growth and a 2,4-D degradation at a high 2,4-D concentration (6 mM) by *Xanthobacter* sp., and AMY *et al.* [4] also suggested a toxicity of 2,4-D at 150 ppm (about 0.2 mM) for the isolates. For *A. eutrophus* JMP134, the inhibition occurs when the 2,4-D concentration is higher than 5 mM [14]. Inhibition effects for the 2,4-D degradation were also observed in our study. Up to 0.7 mM, 2,4-D could be degraded by the resting cells of ANU843p, and 2,4-D concentrations ranging from 1.7 to 4.6 mM obviously inhibited the degradation when a constant cell concentration of ANU843p was used (Fig. 4). The inhibition is usually

considered to result from the toxicity of 2,4-DCP, a product of the 2,4-D degradation pathway [4, 7, 12]. A lower level of 2,4-D (1.7 mM) inhibited the degradation by ANU843p compared to *A. eutrophus* JMP134 (5 mM) despite the same plasmid being involved in the degradation. This may be due to the accumulation of the intermediates of the degradation pathway which are not growth substrates for ANU843p, especially 2,4-DCP.

*Nodulation and* $N_2$ Fixing Ability with ANU843p

When the *R. trifolii* ANU843p was used to inoculate white and subterranean clovers, the capacity to nodulate was maintained, but the ability to fix nitrogen was diminished, particularly for subterranean clover (Tab. 1). This suggests that the transfer of the plasmid pJP4 does not affect the expression of the nodulation genes, but the genes for the expression of the $N_2$ fixing capacity are obviously affected. The reason for the different degrees of effectiveness between white and subterranean clover is unknown.
The transfer and expression of the 2,4-D degrading genes in *Rhizobium trifolii* ANU843p provides prospects for protecting plants from 2,4-D. Legume plants support *Rhizobium* with carbon and energy for growth, which may overcome the possible disadvantage that ANU843p could not grow on 2,4-D as a sole carbon source. Thus, the transconjugant strain ANU843p may degrade 2,4-D without depending on it for growth either in the rhizosphere or inside the root system, while still fixing nitrogen to benefit the plants. These results also suggest a general model for the protection of plants from pesticides and herbicides, with the possibility of transferring different pesticide and herbicide degrading abilities into *Rhizobium* strains.

## Acknowledgements

We thank Jan McIver for discussions and assistance. This work was supported by the research funds from the Rural Industries Research and Development Cooperation and a scholarship from the Australia Development Cooperation Scholarship Scheme.

Received 14 September 1993

## References

[1] Chaudhry, G. R., Chapalamadugu, S.: Biodegradation of Halogenated Organic Compounds. Microbiological Reviews. **55** (1991), 59–79.
[2] Commandeur, L. C. M., Parsons, J. R.: Degradation of Halogenated Aromatic Compounds. Biodegradation **1** (1990), 207–220.
[3] Fry, J. C., Gadd, G. M., Herbert, R. A., Jones, C. W., Watson-craik, I. A.: Microbial Control of Pollution. Forty-eighth Symposium of the Society for General Microbiology held at the University of Cardiff, 1992.
[4] Amy, P. S., Schulke, J. W., Frazier, L. M., Seidler, R. J.: Characterization of Aquatic Bacteria and Cloning of Genes Specifying Partial Degradation of 2,4-Dichlorophenoxyacetic Acid. Appl. Environ. Microbiol. **49** (1985), 1237–1245.
[5] Balajee, S., Mahadevan, A.: Influence of Temperature on Growth, 2,4-Dichlorophenoxyacetic Acid Degradation and Plasmid Transfer in *Azotobacter chroococcum*. Biotech. Techniq. **5** (1991), 55–56.
[6] Chaudhry, G. R., Huang, G. H.: Isolation and Characterization of a New Plasmid from *Flavobacterium* sp. which Carries the Genes for Degradation of 2,4-Dichlorophenoxyacetate. J. Bacteriol. **170** (1988), 3897–3902.
[7] Ditzelmüller, G., Loidl, M., Streichsbier, F.: Isolation and Characterization of a 2,4-Dichlorophenoxyacetic Acid Degrading Soil Bacterium. Appl. Microbiol. Biotechnol. **31** (1989), 93–96.

[8] DON, R. H., PEMBERTON, J. M.: Properties of Six Pesticide Degradation Plasmids Isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145** (1981), 681–686.
[9] EVANS, W. C., SMITH, B. S. W., FERNLEY, H. N., DAVIES, J. I.: Bacterial Metabolism of 2,4-Dichlorophenoxyacetate. Biochem. J. **122** (1971), 543–551.
[10] GREER, C. W., HAWARI, J., SAMSON, R.: Influence of Environmental Factors on 2,4-Dichlorophenoxyacetic Acid Degradation by *Pseudomonas cepacia* Isolated from Peat. Arch. Microbiol. **154** (1990), 317–322.
[11] GREER, L. E., ROBINSON, J. A., SHELTON, D. R.: Kinetic Comparison of Seven Strains of 2,4-Dichlorophenoxyacetic Acid-Degrading Bacteria. Appl. Environ. Microbiol. **58** (1992), 1027–1030.
[12] TYLER, J. E., FINN, R. K.: Growth Rates of a Pseudomonad on 2,4-Dichlorophenoxyacetic Acid and 2,4-Dichlorophenol. Appl. Microbiol. **28** (1974), 181–184.
[13] HAUGLAND, R. A., SCHLEMM, D. J., LYONS III, R. P., SFERRA, P. R., CHAKRABARTY, A. M.: Degradation of the Chlorinated Phenoxyacetate Herbicides 2,4-Dichlorophenoxyacetic Acid and 2,4,5-Trichlorophenoxyacetic Acid by Pure and Mixed Bacterial Cultures. Appl. Environ. Microbiol. **56** (1990), 1357–1362.
[14] PIEPER, D. H., REINEKE, W., ENGESSER, K.-H., KNACKMUSS, H.-J.: Metabolism of 2,4-Dichlorophenoxyacetic Acid, 4-Chloro-2-Methylphenoxyacetic Acid and 2-Methylphenoxyacetic Acid by *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **150** (1988), 95–102.
[15] DON, R. H., WEIGHTMAN, A. J., KNACKMUSS, H. J., TIMMIS, K. N.: Transposon Mutagenesis and Cloning Analysis of the Pathways for Degradation of 2,4-Dichlorophenoxyacetic Acid and 3-Chlorobenzoate in *Alcaligenes eutrophus* JMP134 (pJP4). J. Bacteriol. **161** (1985), 85–90.
[16] DON, R. H., PEMBERTON, J. M.: Genetic and Physical Map of the 2,4-Dichlorophenoxyacetic Acid-Degradative Plasmid pJP4. J. Bacteriol. **161** (1985), 466–468.
[17] KAPHAMMER, B., KUKOR, J. J., OLSEN, R. H.: Regulation of *tfd*CDEF by *tfd*R of the 2,4-Dichlorophenoxyacetic Acid Degradation Plasmid pJP4. J. Bacteriol. **172** (1990), 2280–2286.
[18] PERKINS, E. J., LURQUIN, P. F.: Duplication of a 2,4-Dichlorophenoxyacetic Acid Monooxygenase Gene in *Alcaligenes eutrophus* JMP134 (pJP4). J. Bacteriol. **170** (1988), 5669–5672.
[19] STREBER, W. R., TIMMIS, K. N., ZENK, M. H.: Analysis, Cloning, and High-Level Expression of 2,4-Dichlorophenoxyacetate Monooxygenase Gene *tfd*A of *Alcaligenes eutrophus* JMP134. J. Bacteriol. **169** (1987), 2950–2995.
[20] FRIEDRICH, B., MEYER, M., SCHLEGEL, H. G.: Transfer and Expression of the Herbicide-Degrading Plasmid pJP4 in Aerobic Autotrophic Bacteria. Arch. Microbiol. **134** (1983), 92–97.
[21] INNES, R. W.: A Molecular Genetic Analysis of Host-Range Control in *Rhizobium trifolii*. Thesis. Humboldt State University , 1988.
[22] ROLFE, B. G., GRESSHOFF, P. M., SHINE, J.: Rapid Screening for Symbiotic Mutants of *Rhizobium* and White Clover. Plant Sci. Lett. **19** (1980), 277–284.
[23] VINCENT, J. M.: A Manual for the Practical Study of Root-Nodule Bacteria. I.B.P. Handbook no. 15. Oxford: Blackwell Scientific Publications, 1970.
[24] PLAZINSKI, J., CEN, Y. H., ROLFE, B. G.: General Method for the Identification of Plasmid Species in Fast-Growing Soil Microorganisms. Appl. Environ. Microbiol. **48** (1985), 1001–1003.
[25] SINTON, G. L., FAN, L. T., ERICKSON L. E., LEE, S. M.: Biodegradation of 2,4-D and Related Xenobiotic Compounds. Enzyme Microb. Technol. **8** (1986), 395–403.
[26] SHORT, K. A., DOYLE, J. D., SEIDLER, R. J., STOTZKY, G., OLSEN, R. H.: Effects of 2,4-Dichlorophenol, a Metabolite of a Genetically Engineered Bacterium, and 2,4-Dichlorophenoxyacetate on Some Microorganisms-Mediated Ecological Processes in Soil. Appl. Environ. Microbiol. **57** (1991), 412–418.
[27] KUKOR, J. J., OLSEN, R. H., SIAK, J.-S.: Recruitment of a Chromosomally Encoded Maleylacetate Reductase for Degradation of 2,4-Dichlorophenoxyacetic Acid by Plasmid pJP4. J. Bacteriol. **171** (1989), 3385–3390.