IDENTIFICATION OF SOIL BACTERIA EXPRESSING A SYMBIOTIC PLASMID FROM *RHIZOBIUM LEGUMINOSARUM* BIOVAR TRIFOLII

SIVALINGAM SIVAKUMARAN

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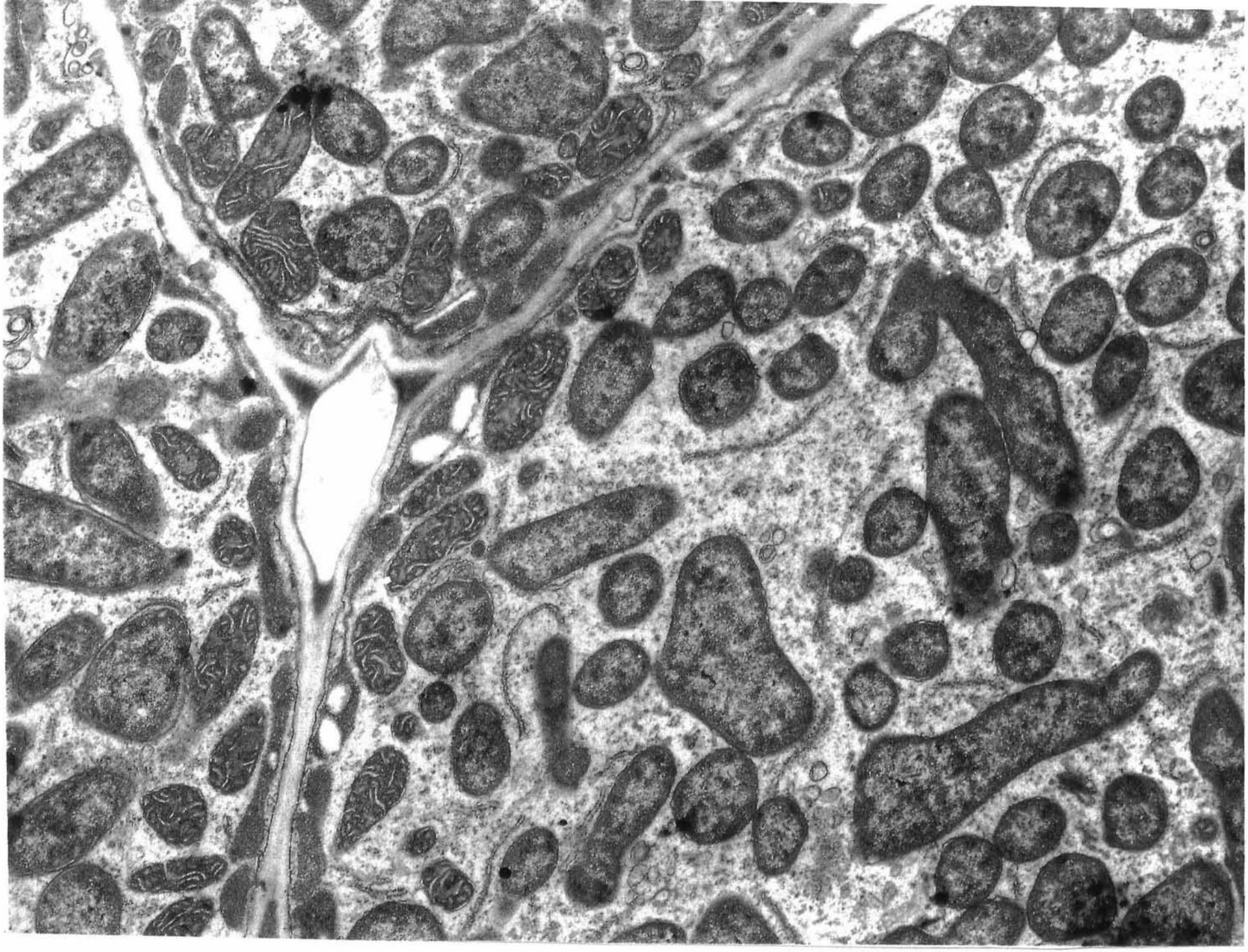
FRONTISPIECE

Electron microscopic section across a nodule formed by the transconjugant soil bacterium KJ30 on white clover (*Trifolium repens*) cultivar Huia (20,900X).



IDENTIFICATION OF SOIL BACTERIA EXPRESSING A SYMBIOTIC PLASMID FROM *RHIZOBIUM LEGUMINOSARUM* BIOVAR TRIFOLII

A thesis presented in partial fulfilment
of the requirements for the degree of
Doctor of Phy losophy in Microbiology
at Massey University, Palmerston North, New Zealand

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1994

DEDICATION

.....to our parents for their love, care and encouragement.

...the sure and definite determination (of species of bacteria) requires so much time, so much acumen of eye and judgement, so much perseverance and patience that there is hardly anything else so difficult---Mueller

ACKNOWLEDGMENT

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Brion and Audrey Jarvis for helping me to settle in during my early stages of my Ph.D. studies and making me an independent and a mature person.

Finally thanks to all those I have missed out!.

ERRATUM

Page	Line	Incorrect text	Correct text
vi	30	plants inoculated	plants were inoculated
3	4	on fixed nitrogen	symbiotically fixed nitrogen
7	5	bacteroids lack	bacteroids which lack
7	11	represent	representing
9	18	possess	possesses
13	26	has	have
15	1	dendeogram	dendogram
16	25	example	examples
16	26	and will	and these will
18	27	competition of	competition with
24	6	to cured a	to a cured
32	31	is	are

ABSTRACT

The present study concerns the identification of soil bacteria, which could not in their isolated state nodulate white clover, but which could accept a transconjugant plasmid encoding the *nod* gene, and subsequently establish a symbiosis with white clover leading to nodulation. This follows earlier studies intended to characterize non-symbiotic *Rhizobium* strains from the soil. However, whilst these studies specifically examined the potential of non-symbiotic *Rhizobium* strains to nodulate, the present work was developed to examine the potential of any Gram negative soil bacteria to express a transconjugant *nod* plasmid.

A collection of soil bacteria from four different soil types namely (i) Ramiha silt loam, (ii) Tokomaru silt loam, (iii) Kairanga silt loam under white clover-ryegrass pastures and (iv) Manawatu sandy loam (a fallow land with shrubs of *Lupinus* sp.) were isolated and purified. A total of 100 strains of soil bacteria with varying colony morphology were isolated and maintained on media not selective for rhizobia. Each was checked for its ability to nodulate white clover (*Trifolium repens*) cultivar Grasslands Huia. Only four strains nodulated. Conjugation experiments were set up for non-nodulating strains using *Escherichia coli* strain PN200 which contained plasmid pPN1 (pRtr514a::R68.45). A total of 12 soil isolates out of 100 crosses made (12%) formed nodules on white clover, and one strain KJ1 formed transconjugants on a selective antibiotic plate but failed to nodulate white clover. The bacteria accepting and expressing pPN1 were from several soil types including leached, low phosphorous (P) and low pH soil such as Ramiha silt loam.

We showed that eight soil strains formed transconjugants with a mean frequency of transfer of 2.91×10^{-5}. Seven out of these eight strains nodulated white clover. We could not calculate the frequency of transfer for the remaining five isolates, as antibiotic resistant recipients could not be obtained but the transconjugant mixture was inoculated on clover seedlings and all the five strains nodulated white clover.

In our experiments nodulation by transconjugant soil bacteria was verified by plant tests in nitrogen-deficient medium. True nodules formed on white clover seedlings, and on the positive control *Rhizobium leguminosarum* biovar trifolii strain ICMP2163. The negative control plants inoculated with sterile water, *Escherichia coli* strains PN200 containing pPN1 or *E. coli* strain ATCC9637 and the recipient soil bacteria did

not nodulate. It was concluded that nodule formation was due to the transfer of pRtr514a by conjugation.

Eckhardt gels showed that the transconjugants contained different parts of the co-integrate. Strains KJ1 and KJ3 contained R68.45 only, strains KJ13, KJ19, KJ23, KJ26, KJ30 and KJ44 contained pPN1 and R68.45 whilst strains KJ5, KJ17, KJ27, KJ57, KJ203 and PN165 contained pPN1.

Microtome sections of nodule tissue were examined by light and electron microscopy to determine the distribution of infected plant cells and verify that these cells contained bacteroids enclosed in plant cell membranes. The nodule cells formed by the inoculant *R. leguminosarum* biovar trifolii strain ICMP2163 and most cells of all transconjugants were filled with bacteroids. A few nodule cells formed by the transconjugants were devoid of bacteroids.

Total genomic DNA was extracted from each of the transconjugants isolated from the nodules, and from a selective antibiotic plate for strain KJ1, and digested with restriction endonucleases. The fragments were separated by gel electrophoresis, transferred to nylon membrane and probed with an amplified 590 bp *nod*A sequence. Eleven strains of transconjugant soil bacteria gave a hybridization signal at 11.7 Kb with the *nod*A probe. However KJ1 and KJ3 failed to hybridize with the 590 bp *nod*A sequence. KJ1 did not nodulate but formed transconjugants on selective antibiotic plates whereas KJ3 nodulated white clover. The failure to detect *nod* genes in KJ3 may have been due to a loss of pPN1 during sub-culture. Overall the hybridization results confirmed that soil harbours non-nodulating soil bacteria which can maintain symbiotic genes and symbiotic plasmids.

Four methods were used for the identification of soil bacteria expressing pSym. These were (i) rRNA fingerprinting, (ii) 16S rRNA sequence analysis, (iii) DNA-DNA hybridization, and (iv) Total fatty acid analysis. Initially the transconjugants were characterized by rRNA fingerprinting. However this approach was insufficient to identify all isolates. 16S rRNA sequence analysis and DNA-DNA hybridization were subsequently used. These comparisons were more informative and all strains were identified as *Rhizobium* or *Agrobacterium* species. The fatty acid content of the strains was analyzed by gas-liquid chromatography. A comparison of the species names assigned by CFA with those assigned by DNA analyses showed only 50% agreement. These observations are discussed in relation to the phylogenetic distinctiveness of *Agrobacterium* and *Rhizobium*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES