Figure 2. Mature lac insect females on pumpkin secreting white wax filaments.

Table 1. Various attributes of lac insect life cycle showing normal behaviour on pumpkin.

Strain used	Date of. propagation	Male emergence (after settlement)	Total time taken (in days) to mature	Mean	No. of insects studied
Kusmi	14 July '90	7–8 weeks	142–160	153	83
Rangeeni	29 June '90	6–7 weeks	120–137	124	94

quality and quantity of lac. Another secondary objective of such study is to artificially rear lac insects on synthetic diet in laboratory to conduct certain studies on genetical, physiological and toxicological aspects of lac insect and its major predators; (ii) make ecological studies in controlled physical environment more convenient; (iii) make lac insect amenable to certain experimentation techniques; (iv) save much of labour and money as it requires practically nothing towards its maintenance.

Efforts are being made at this institute to standardize the rearing technique for further studies.

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Construction of a gene bank of *Cicer-Rhizobium*

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A gene bank of *Cicer-Rhizobium* has been constructed using a broad host range conjugally transferable cosmid vector pLAFR1. The random analysis of clones from the gene bank showed average insert size of 21.1 kb and a high degree of randomness. As an initial test, clones were isolated carrying *str* gene of strain Rcd301 (Str^R) using F75 (Str^S) as the recipient. The frequency of transfer of clones of the bank from *E. coli* to *Cicer-Rhizobium* was 4×10^{-4} and of Str^R transconjugants was 1.2×10^{-6} per recipient cell. The maintenance of cosmid clones was confirmed by growing transconjugants without selection and also passing through chickpea nodules.

CICER-RHIZOBIUM is the soil bacterium that forms symbiotic nodules on the roots of chickpea (*Cicer arietinum*) plants and fixes atmospheric nitrogen there. Cross inoculation group specificity studies on chickpea and its nodule bacteria have shown *Cicer-Rhizobium* as a distinct group than known groups of alfalfa, clover, pea, bean, soybean, lupin-lotus and cowpea miscellany[1]. India is the premier chickpea-growing country accounting for 76% of the total area and production in the world[2]. Within the country, chickpea is the predominant pulse crop covering 30% of the area and 37% of the production of all pulses grown. In spite of this fact India has been harvesting only one third of the productivity potential at the national level[3].

Developing effective and competitive strains of *Rhizobium* as a biofertilizer is a desirable step to improve the pulse production in general at low cost[4]. Present day varieties of chickpea are adapted for marginal conditions, including low soil fertility, minimum agronomic inputs and poor crop management. Under field conditions[5] seed inoculation with chickpea *Rhizobium* has demonstrated a significant increase in grain yield of chickpea from 4 to 67%. Understanding the genetics of symbiotic and adaptive functions in chickpea *Rhizobium* would be, therefore, a pre-requisite for its directed improvement through genetic manipulations and recombinant DNA approach. Such studies would be facilitated if the complete gene bank of an effective *Rhizobium* strain is available. A cosmid gene bank of a wild type *Cicer-Rhizobium* strain Rcd301 was constructed on a broad host range cosmid vector pLAFR1[6]. The construction of gene bank and its use in isolation of recombinant cosmids containing Str^R gene is described.

Bacterial strains and growth media. Strains of *E. coli* and *Rhizobium* used in the study are listed in Table 1.

Table 1. Bacterial strains and plasmids used.

Strain/plasmid	Relevant characteristics	Reference
E. coli		
HB101	*recA, hsdR, hsdM, pro, leu, Str*[R]	19
HB101 (pLAFR1)	Contains pLAFR1	6
HB101 (pRK290)	Contains pRK290	14
HB101 (pRK2013)	Contains pRK2013	14
C2110 nal	*nal, his, rha, polA*	20
HB101 (pLAR1-Rc gene bank)	Contains EcoRI gene bank of Rcd301 on pLAFR1	Present work
Cicer-Rhizobium		
F75	Wild type	5
Rcd301	Spontaneous Str[R] derivative of F75	Present work
R. meliloti		
102F34 nal	Wild type	14
Rmd201	Wild type	9
Plasmids		
pLAFR1	Wide host range cosmid vector, Tet[R], *ori*, (RK2)	6
pRK290	Wide host range plasmid vector, Tet[R], *ori* (RK2)	14
pRK2013	Helper plasmid, Kan[R], *tra* (RK2), *ori* (colE1)	15

E. coli cells were routinely cultured on LB medium[7]. LB was supplemented with 0.2% maltose for growing recipient cells for cosmid transduction[8]. Complex media used for growing *Rhizobium* were MSY[9] and TYM (bactotryptone, 5 g; yeast extract, 0.5 g; mannitol 10 g and $CaCl_2.2H_2O$ 1 mM l^{-1}). Minimal medium used for *Rhizobium* was as described by Sikka and Kumar[10]. Difco agar was used at 1.6% for pouring plates. Both *E. coli* and *Rhizobium* were grown at 30°C. Antibiotics and drugs were added at the following concentrations (μg/ml): kanamycin (50), tetracycline (10 for *E. coli*, 5 for *Rhizobium*), streptomycin (100) and nalidixic acid (10).

DNA isolations. Total DNA from *Cicer-Rhizobium* was obtained from one litre of Rcd301 culture grown for 24 h in TYM broth (approximately 2×10^8 cells ml^{-1}). Washed cells were resuspended in TE (50 mM Tris–HCl/20 mM EDTA pH 8.0) and lysed with predigested pronase (500 μg/ml) and sarkosyl (1%) for 60 min at 50°C. DNA was purified by equilibrium centrifugation twice in CsCl/ethidium bromide density gradient. Cosmid pLAFR1 DNA from *E. coli* HB101 was isolated by modified Birnboim and Doly[11] procedure and then purified by CsCl/ethidium bromide density gradient. DNA from the gradient in both cases was extracted with butanol several times and then dialysed against DNA storage buffer (DSB: 6 mM Tris pH 7.4, 10 mM NaCl, 0.1 mM EDTA) for 24 h at room temperature. The quantity and purity of DNA samples were confirmed by taking OD at 260 and 280 nm on spectrophotometer[12].

Partial digestion and size fractionation. Conditions for partial digestion of Rcd301 DNA to yield 17–23 kb

EcoRI fragments were established using 15 μg DNA and a series of EcoRI concentrations (1, 0.5, 0.25, 0.125, 0.0625 units per μg DNA). Large-scale partial digestion was then carried out for 600 μg of DNA using EcoRI at 0.125 units per μg DNA. The partial digest was phenol-extracted twice and ethanol precipitated. This DNA (150 μg) was heated briefly at 68°C and layered onto 36 ml, 10–40% sucrose gradient in DSB. It was centrifuged for 26 h at 24,000 rpm at 20°C in SW 27 rotor. Fractions (0.5 ml) were collected and aliquots monitored on 0.4% agarose gel. Fraction numbers 23 and 24 containing predominantly 17–23 kb fragments were pooled and used for construction of gene bank.

Construction of recombinant cosmid clones. Vector pLAFR1 DNA was exhaustively digested with EcoRI and then treated with calf intestine alkaline phosphatase (0.02 units per μg). The size fractionated *Rhizobium* DNA was ligated to this vector DNA in equimolar ratio (2 μg DNA of each) and ligation test carried out on 0.4% agarose gel using Lambda uncut DNA as control.

Packaging of cosmid clones and transduction. Amersham kit was employed for *in vitro* packaging of recombinant cosmid molecules. The procedure of Hohn[13], using lysogenic *E. coli* BHB 2690 *(Dam)* and BHB 2688 *(Eam)*, was followed. For packaging 0.5 μg of ligated DNA was used and finally suspended in 0.5 ml of phage dilution buffer (10 mM Tris–HCl pH 7.4; 10 mM $MgSO_4$, 0.01% gelatin) and maintained at 4°C on chloroform. HB101 cells were grown overnight at 37°C in LB containing 0.2% maltose and then infected with package mix (10 μl per 0.2 ml cells). The plating was done on LB agar containing tetracycline (10 μg ml^{-1}). About 45,000 colonies that developed were pooled to constitute the gene bank of Rcd301. The average insert size and insertion efficiency was ascertained by analysing a dozen of randomly picked clones (Figure 1).

Bacterial matings. Matings were performed by mixing approximately 10^9 cells of each donor, helper and recipient strains and filtering the suspension through 0.45 μm millipore filters. The filters were incubated at 30°C on MSY agar plates overnight. The cells were suspended in MSY broth and plated on selective medium plates.

Cloning vector. In order to have a suitable vector for introducing genes and stable maintenance in *Cicer-Rhizobium* cells, initial experiments were performed with broad host range vectors pRK290[14] and pLAFR1[6] employing pRK2013[15] as helper plasmid in triparental matings. The transfer frequency of both the vectors was about 10^{-4} per recipient cell into *Rhizobium* strains F75
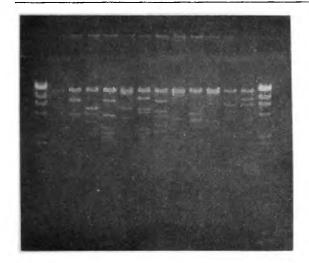
Figure 1. *Eco*RI restriction pattern of cosmid clones of the gene bank of *Cicer-Rhizobium* Rcd301. The first and last lanes show the fragments generated by *Hind*III digestion of lambda DNA (top to bottom 23.3, 9.5, 6.4, 4.2, 2.2 and 1.8 kb). Remaining lanes have *Eco*RI digests of the randomly picked 12 clones from the clonal library. The average size of the inserts was estimated as 21.1 kb.

and Rcd301 (Table 2). Since pLAFR1 has advantage of *in vitro* packaging into lambda heads, it was used as cloning vector for constructing the *Cicer-Rhizobium* gene bank.

Gene bank of Cicer-Rhizobium DNA. Total DNA of *Cicer-Rhizobium* strain Rcd301 was partially digested with *Eco*RI and fractionated on sucrose gradient to yield 17–23 kbar fragments (desired insert size). The cosmid vector (pLAFR1) DNA was also linearized using *Eco*RI and treated with alkaline phosphatase to prevent self-ligation. Ligation was carried out with equimolar ratio (2 μg each of vector and inserts) since vector and insert had approximately similar size[12]. Ligated DNA was packaged into lambda using Amersham kit and plated on *E. coli* HB101 to obtain approximately 45,000 tetracycline-resistant colonies to

Table 2. Conjugal transfer frequencies of the vector and recombinant clones using binary vehicle system*.

Donor	Recipient	Tet^R exconjugants per recipient	Tet^R Str^R exconjugants per recipient
HB 101 (pRK290)	F75	4.56×10^{-4}	–
	Rcd301	4.40×10^{-4}	–
HB101 (pLAFR1)	F75	4.38×10^{-4}	–
	Rcd301	4.45×10^{-4}	–
HB101 (pLAFR1-bank)	C2110 nal	2.25×10^{-1}	–
	102F34 nal	6.70×10^{-2}	–
	Rmd201	5.85×10^{-2}	–
	F75	4.11×10^{-4}	1.21×10^{-6}

*HB101 (pRK2013) was used as helper strain in these triparental matings.

constitute the gene bank. Analysis of randomly picked 12 clones from the gene bank using *Eco*RI digestion showed all the clones possessing inserts (Figure 1) and the average insert size determined on this basis was observed to be 21.1 kbar. This indicates a very high efficiency of the library considering the properties in terms of the insertion frequency and randomness of the clones.

Efficiency of transfer. The cosmid vector pLAFR1 used for cloning lacks the DNA region necessary for conjugal transfer (Tra). Thus it can be mobilized by a helper plasmid pRK2013 providing transfer functions in *trans*. This constitutes the binary vehicle system for efficient mobilization of cloned fragments from *E. coli* into a variety of gram-negative bacteria[6,14].

The relative frequencies with which the cosmid clones can be transferred using a binary vehicle system[6,14] into *E. coli*, *R. meliloti* and *Cicer-Rhizobium* are listed in Table 2. Triparental matings were carried out using pRK2013 as helper plasmid. There was efficient transfer of the cosmid into rhizobia from *E. coli* and within *E. coli* strains. But relative efficiency of the transfer was highest within *E. coli* followed by transfer into *R. meliloti* and then *Cicer-Rhizobium*. In *Cicer-Rhizobium* the transfer frequency was of the order of about 4×10^{-4} while in *R. meliloti* it was about 10^{-2}. The frequency of transfer in *Cicer-Rhizobium* is comparable to the transfer of *R. japonicum* gene bank from *E. coli* to *Rhizobium* as observed by Cantrell *et al.*[16] using pLAFR1 cosmid vector.

Isolation and stability of complementary clones. In order to have a preliminary test of the gene bank, streptomycin-resistance (Str^R) property of the derivative strain Rcd301 was used. Streptomycin-sensitive parent strain F75 was used as recipient in triparental matings with clone bank *en masse* as donor. Tetracycline-resistant (Tet^R) and streptomycin-resistant (Str^R) exconjugants were selected on minimal medium. Tet^R colonies appeared at frequencies of 10^{-3} to 10^{-4} per recipient cell, while Tet^R Str^R colonies were obtained at 10^{-6} per recipient cell or 10^{-3} per transconjugant. The frequency of the Str^R colonies was significantly higher than the spontaneous mutation rate for Str^R colonies (about 10^{-10}), demonstrating that the Str^R phenotype was because of complementing clone from the gene bank.

The extent of stability of cosmid clones was tested by growing the cells on non-selective medium for about 25 generations and also passing through nodules. When grown without selection pressure, the loss of cosmid appeared less than 1% per generation. Similar results were observed even when bacteria were reisolated from nodules infected by exconjugants.

Thus, a cosmid gene bank of *Cicer-Rhizobium* has

been constructed which is mobilizable from *E. coli* into rhizobia using binary vector system. Assuming size of *Rhizobium* chromosome similar to that of *E. coli* (about 4200 kb), average insert size being 21.1 kb, the gene bank should represent all the genes of *Cicer-Rhizobium* with 98% chance according to the formula of Clarke and Carbon[17] $[P = 1 - (1-f)^N]$. In this formula, the probability (P) that a given unique DNA sequence is present in a collection of N transformant (45,000 in this study) colonies, is given by above expression, wherein, f is fraction of the total genome represented by average fragment (21.1 kbar here). This genomic library will be useful in dissecting various symbiotic and adaptive functons in these rhizobia where little is known about the genetic system involved. Attempts are being made to identify the clones containing genes involved in symbiotic nitrogen fixation. Such studies have been fruitful in various rhizobia like *R. meliloti*[9,14], *R. japonicum*[16] and *R. leguminosarum*[18].

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BAMBOO INFORMATION CENTRE-INDIA

A Bamboo Information Centre has been established in Kerala Forest Research Institute, Thrissur, in cooperation with the International Development Research Centre (IDRC), Canada. The centre is functioning since July 1989. Its aim is to collect all published literature on bamboos from Asia. It also collects information about scientists working on bamboos and ongoing bamboo research projects, and provides search services from its computerized database backed up with document delivery service. The Centre brings out a number of publications, including a half-yearly BIC Bulletin containing abstracts of literature, articles, news and information about scientists and research projects; information bulletins on various aspects of bamboo; and a compendium on Indian bamboos. We encourage persons and institutions interested in bamboo-related information to contact the centre for obtaining its services and for inclusion of details about scientists and research projects and for publishing articles on bamboos.

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