DEVELOPMENT OF A GENETIC SYSTEM IN *RHIZOBIUM MELILOTI*

by

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ABSTRACT

This thesis is the development of a genetic system in the symbiotic nitrogen fixing bacterium *Rhizobium meliloti*. Drug resistant and auxotrophic mutants of the effective strain Rm2011 were first isolated. The genetic system is based on conjugation by the drug resistance factor RP4. During mating RP4 can promote chromosome mobilization to produce recombinant progeny. By carrying out crosses between multiply marked derivatives of Rm2011, a circular linkage map of *R. meliloti* was constructed. This is the first genetic transfer system and resulting linkage map of *Rhizobium meliloti*.

During this study, a spontaneous Hfr was isolated. In an attempt to generate Hfrs, a derivative of RP4 that is temperature sensitive for replication was found. Recombinants between RP4 and F'ColVB*trp* were selected, which have the mating properties of RP4. One of these has been used to introduce λh80 into *R. meliloti*.

Thesis Supervisor: Ethan Signer, Associate Professor of Microbiology

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GENERAL INTRODUCTION

Rhizobia fix atmospheric nitrogen in the nodules they form on the roots of legumes. This rhizobia-legume symbiotic relationship is the main means by which biological nitrogen fixation is harnessed for agricultural production. Not only are the grain legumes (pulses) a good source of protein but when they are included in crop rotation practices, there is a net increase of available fixed nitrogen in the soil.

Even though rhizobia have been studied for many years, there has been no detailed genetic analysis of the rhizobia-legume relationship. This has been mainly due to the lack of a suitable genetic system in a nodulating strain of Rhizobium.

This thesis was begun with the goal of developing a usable genetic system, and I describe here the results of this effort. I have developed a genetic system in Rhizobium meliloti, Rm2011 that is based on conjugation. During mating the drug resistance factor RP4 can mobilize the bacterial chromosome to produce recombinant progeny. Using this system I have constructed a linkage map of R. meliloti.

This thesis is the necessary first step in the genetic analysis of the rhizobia-legume symbiosis--development of the genetic system.

A number of reports have described genetic transfer in Rhizobium. As early as 1953, Balassa (1) reported transformation among three species, and there have been occasional reports since then (2-5). It was not until just recently that linkage was reported using transformation in R. japonicum (6).

Sik and Oroz (7) have reported specialized transduction of cys in

R. meliloti. Generalized transduction has been shown by Kowalski, (8), also in R. meliloti.

There have been many reports of conjugation in Rhizobium. Higashi (10) reported the transfer of nodulation specificity between two different species and Lorkiewica (11) described the transfer of auxotrophic markers in R. trifolii. There have been no subsequent reports in either case. Heumann (12) reported mating and a circular linkage map in a strain reported to be R. lupini. However, this strain no longer nodulated (13) and unlike other rhizobia forms colored colonies on agar plates, which makes this difficult to evaluate.

The genetic system I describe here for R. meliloti is based on conjugation promoted by the drug resistance factor RP4. Using this system I have carried out a number of crosses between multiply marked derivatives of the parental strain of R. meliloti. By scoring the selected exconjugants for cotransfer of nonselected markers, I have constructed a linkage map of R. meliloti. This is the first genetic system and resulting circular linkage map of a nodulating strain of Rhizobium.

I have presented preliminary reports of this work (14, 15) and am submitting a paper describing the system. Beringer has just reported a similar system using another P-type R factor, R68-45, in R. leguminosarum (16).

II. METHODS

A. CHEMICALS

1.	Lysozyme	Worthington Biochem. Corp.
2.	Carbenicillin	Pfizer
3.	Spectinomycin	Upjohn
4.	Rifampin	Calbiochem
5.	Tetracycline	"
6.	5-Fluorouracil	"
7.	Chloramphenicol	Sigma
8.	Kanamycin	"
9.	Neomycin	"
10.	Novobiocin	"
11.	Penicillin G	"
12.	Polymyxin B	"
13.	Streptomycin	"

LB broth: Per liter, 10g tryptone, 5g yeast extract, 5g NaCl, 4ml of 1N NaOH

LB agar: Same as above except only 1ml of 1N NaOH and 15g agar

Soft agar: Same as LB with 6.5g agar

LBCa: Lb broth plus 0.5mM $CaCl_2$

M9 : Per liter, 5.8g Na_2HPO_4, 3g KH_2PO_4, 0.5 NaCl, 1g NH_4Cl and 1mM $MgSO_4$ added after autoclaving

M9 agar: Same as above with 15g agar

GB_1M9: For E. coli M9 is supplemented with 0.2% Glucose and 0.5ug/ml thiamine

SbM9: For Rm2011 M9 is supplemented with 0.2% Sucrose and 0.5ug/ml biotin

All minimal media are supplemented with 20ug/ml of amino acids or bases whenever needed

SAY: Per liter, 0.5g K_2HPO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 0.5mM $CaCl_2$, 10g yeast extract, 2g Sucrose

SAY agar: Same as above with 15g agar

SAY top: Same as SAY with 6.5g agar

AA agar: Same as SAY with 120mg cycloheximide, 60mg pentachloronitrobenzene, 1.5ml of 1% Congo Red solution

Nodulation: Per liter, 1.0g Ca_2HPO_4, 0.2g K_2HPO_4, 0.2g $MgSO \cdot 7H_2O$ 0.2g NaCl, 0.1g $FeCl_3$, Adjust to pH7.0

STAB agar: Per liter, 10g Nutrient Broth, 8g NaCl, 20mg cysteine 6g agar

Bacterial strains are listed in Table II-C-1. E. coli strains carrying R factors were kindly donated by G. Jacoby at Mass. Genaeral Hospital. These strains are shown in Table III-B-3. All E. coli strains used were from the collection of Ethan Signer.

The parental Rhizobium meliloti strain,Rm2011 str3, was donated by Jean Denarie in Versailles, France. This strain is a streptomycin resistant mutant of R. meliloti SU47 from P. J. Nutman at Rothamsted Experimental Station, England. I isolated all of the derivatives of Rm2011 described in this this.

Phage strains were from the collection of Ethan Signer except where otherwise noted. Phage are listed in Table II-C-2.

II. C. 1 RHIZOBIUM MELILOTI STRAINS

Rm2011 str3 parental wild type

All are derivates of Rm2011 str3

Rm2011	cys11	Rm3300	trp33	spc1		
Rm2012	gly12	Rm3330	trp33	spc1	rif1	
Rm2013	ilv13	Rm3339	trp33	his39	spc1	rif1
Rm2014	ade14	Rm3341		"		arg41
Rm2018	his18	Rm3342		"		cys42
Rm2024	met24	Rm3343		"		gly43
Rm2025	his25	Rm3344		"		pan44
Rm2026	leu26					
Rm2027	ilv27	Rm3346		"		cys,gly
Rm2028	met28	Rm3347		"		ser,gly
Rm2029	pan29	Rm3348		"		ilv48
Rm2031	ade31	Rm3349		"		pyr49
Rm2032	cho32	Rm3350		"		cys50
Rm2049	his18 rif2	Rm3351		"		aro51
Rm2079	his18 leu79 rif2	Rm3353		"		leu53
Rm2080	cyt80 pyr80	Rm3354		"		phe54
		Rm3355		"		lys55
		Rm3356		"		met56
		Rm3357		"		leu53, nov57
		Rm3358	trp33	nov57	spc1 rif1	leu53

Rm3363	his39	pan44	gly12	rif1 / RP4 Hfr				
Rm3373	trp33	his39	leu53	spc1	rif1	nov57	nal73	
Rm3374	"	"	pyr49	"	"	nov59	ery74	
Rm3375	"	"	leu53	"	"	nov57	gly75	
Rm3376	"	"	"	arg76	spc1	rif1	nov57	
Rm3377	"	"	"	ilv77	"	"	"	
Rm3378	"	"	"	phe78	"	"	"	
Rm3379	"	"	"	aro79	"	"	"	
Rm3381	"	"	pyr49	lys81	"	"	nov59	
Rm3382	"	"	"	phe82	"	"	"	
Rm3383	"	"	"	aro83	"	"	"	
Rm3386	"	"	"	lys55	"	"	"	pmx86
Rm3388	"	"	leu53	arg76	"	"	nov57	pmx88
Rm3389	"	"	pyr49		"	"	nov59	pmx89
Rm3390	"	"	pan44		"	"	nov90	

Rm2100/RP4 gly12 (ATK)

Rm2101/RP4 " pmx1 (ATK)

Rm2102/RP4 " ery2 (ATK)

Rm2103/RP4 " van1 (ATK)

II. C. 2 PHAGE STRAINS

λc^{+}
$\lambda_{cI857}{}^{h80}$
$\phi 80_{\ v5}$

P1Cm, clri100	Richard Goldberg
P1 vir	"
P1kc	"
T5	"
T7	"
P22	Nancy Kleckner
P22Tet	"
λ_{cI60}	"
MP1-3	Soil isolate

E. coli

E. coli strains are routinely grown at 37°C except for temperature sensitive lysogens which are grown at 30°C. Rich medium is LB and minimal medium is M9 supplemented with 0.2% Glucose, 0.5ug/ml B_1, and 20ug/ml of amino acids or bases when needed.

R. meliloti

Rm2011 is a fast growing strain of Rhizobium that unlike most strains grows well on standard E. coli media. LB is used for rich medium and minimal medium is M9 supplemented with 0.2% Sucrose, o.5ug/ml biotin, and 20ug/ml of amino acids or bases when needed. Rm2011 has a growth range of 22° to 39°C. These bacteria are routinely grown at 32°C.

The doubling time of Rm2011 in rich medium is 2½ to 3 hours and single colonies appear on LB agar within 3 days. In minimal medium the doubling time is 6 hours and 5 days are required to see colonies on minimal plates.

SAY medium is a standard Rhizobium growth medium (17) which is used for phage isolation and growth. With the addition of a dye and antibiotics this medium can be used to isolate rhizobia from the soil (AA medium) as described by Grahm (18). The rhizobia are recognized by their white mucoid colonies which do not take up the dye.

II. E. STORAGE

Rhizobium have traditionally been stored in refrigerated slants. These slants must be reinoculated every month as described by Vincent (17). There is a constant selection that occurs each time the rhizobia are inoculated into a new slant. This may account for the loss of infectivity in those strains that have been kept under laboratory conditions for many years.

I have found that Rm2011 can be stored in stabs used routinely for *E.coli*. This has eliminated the need to repeatedly transfer the rhizobia to new slants. Rm2011 has shown no loss of nodulation ability after being kept in a stab for five years.

II. F. NODULATION

Nodulation is carried out as described by Vincent (17). Alfalfa seeds are surface-sterilized by soaking in one half strength Chlorox for 15 minutes, then washed repeatedly with sterilized water. The seed are then placed in a growth chamber prepared as follows.

A piece of #29 Whatman balck filter paper is rolled to form a cylinder that can be pushed to the bottom of a 20 x 150 mm test tube so that it stands on edge. Three seeds are placed between the paper and the tube wall. Eight ml of Nodulation medium are then added and a glass cap is placed over the top of the tube.

The seeds germinate within four days and one week later their roots extend to the bottom of the tube. The growth chambers are placed in a window for daylight and also supplemented with Sylvania Gro-lite at 1000 lux for a 14 hr. day. The average temperature is $26^{o}C$. The roots are kept dark.

The plants are then inoculated with about 10^{8} rhizobia that have been washed and resuspended in sterile water. One week after inoculation nodules begin to appear, and within a month there are several nodules on each plant.

Effectiveness can be monitored within 2 months. Since the plants are growing in nitrogen-free medium, uninoculated controls become stunted and chlorotic. Plants inoculated with effective Rm2011 are lush, green and healthy.

II. G. MUTAGENESIS

Introduction

I first determined the optimal conditions for mutagensis by measuring the survival versus mutagenic rate for EMS, NTG, and UV irradiation. Controls were removed prior to mutagensis and carried through the rest of

the procedures in parallel with the mutagenized cells. Survival was determined by comparing the number of rif (rifampin) resistant colonies[17] found on LBrif plates to the unmutagenized control. Both the controls and the experimental cells were grown overnight before being plated on LBrif to allow the expression of the rif resistant phenotype. The methods are essentially those described by Miller (19).

EMS (ethyl methyl sulfonate)

Cells were grown in LB to saturation, washed twice and resuspended in one half the original volume of Tris-saline pH 7.6. A control aliquot was removed, and two drops per ml of EMS was added to the cells. The mixture was incubated at 30°C without shaking. At various times aliquots were removed, washed with 0.02M $Na_2S_2O_3$, titered, diluted ten fold into LB, and allowed to grow overnight as described above. The results are shown in Table II-G.

NTG

Cells were grown to log phase, washed twice and resuspended in the original volume of 0.01M Citrate Buffer pH 5.5. NTG from frozen stock (2.5mg/ml) was added to a final concentration of 100ug/ml. The cells were incubated at 30°C without shaking. At various times aliquots were removed, washed with Phosphate Buffer pH 7.0, and resuspended in LB. Survival and mutagenesis were measured as described above. The results are shown in Table II-G.

UV Irradiation

Cells were grown to log phase, washed twice and resuspended in saline. The cells were then irradiated with a germicidal U.V. lamp at 5 ergs/min. At various times aliquots were removed, titered, and diluted into LB. The results are shown in Table II-G.

TABLE II-G

COMPARATIVE MUTAGENSIS

EMS

Time (min)	0	30	60	90	120
Survival (5)	100	90	25	1	0.1
Rif resistant colonies	4	30	1200	1000	500
Increase (X)	1	8	300	250	125

NTG

Time (min.)	0	15	30	60	90
Survival (%)	100	23	11	1	0.5
Rif resistant colonies	7	700	2000	1100	-
Increase (x)	1	100	267	167	-

UV

Time (sec.)	0	15	30	45	60
Survival (%)	100	25	3.4	0.1	0.004
Rif resistant colonies	10	200	1000	250	-
Increase (X)	1	20	100	25	-

II. H. MUTANTS

1. Drug resistant

Mutants resistant to various antibiotics and antimetabolites have been isolated. These mutants are useful in counterselection schemes and the mutations themselves are markers that can be mapped in genetic studies.

In order to isolate mutants of Rm2011 resistant to drugs and antimetabolites it was first necessary to test this strain for sensitivity to various agents. Antibiotics were tested using either Pfizer Test Discs (small paper discs impregnated with antibiotic) or a few grains of powdered antibiotic placed on a lawn of Rm2011 growing on LB agar. Antimetabolites and analogs were tested by placing the powdered agent on Rm2011 growing on minimal agar. The results are shown in Table II-H.

Once those agents which are toxic to Rm2011 were found, they were further tested for the level at which mutants could be isolated. This was done by two fold dilutions starting at 500ug/ml. I chose a level at which a log phase culture would give individual resistant colonies and no background when plated out. The concentrations at which the mutants were isolated are listed beside each agent in Table II-H.

TABLE II-H

DRUG AND ANTIMETABOLITE SENSITIVITY

DRUG		ANTIMETABOLITE	
Trimethoprin	R	D-histidine	R
Streptolydigan	R	D-serine	R
Puromycin	R	L-valine	R
Sulfathiazole	R	5-methly tryptophane	R
Cephalosporin	R	methyl anlanine	R
Mendalamine	R	thiaolyl anlanine	R
Cloxicillin	R	p-fluoro-phenyl alanine	R
Penicillin	S 50ug/ml	methyl aspartic acid	R
Chloramphenicol	S 50ug/ml	azaxanthine	R
Rifampin	S 50ug/ml	azaguanine	R
Spectinomycin	S 100ug/ml	azahypoxanthine	R
Ampicillin	S 40ug/ml	azauracil	R
Novobiocin	S 25ug/ml	5-fluoro uracil	S 100ug/ml
Polymyxin B	S 2ug/ml	deoxygalactose	R
Gentamicin	S 50ug/ml	ethionine	S 100 ug/ml
D-cycloserine	R		
Vancomycin	S 50ug/ml		
Bacitracin	R		
Erythromycin	S 50ug/ml		
Nalacixic Acid	S 50ug/ml		
Kanamycin	S 100ug/ml		

S--sensitive
R--resistant

II. H. 2. Auxotrophic mutants

Various amino acid and base requiring auxotrophic mutants have been isolated by a modification of the Penicillin enrichment method described by Davis (20). Although Rm2011 was sensitive to Amp and Pen G (see II-H), these drugs were ineffective in enrichment procedures since they gave only 10 fold killing in minimal media. Carbenicillin (Pfizer) a synthetic derivative of penicillin was effective and could be used to enrich for auxotrophs.

Log phase mutagenized cells grown in LB were washed twice and resuspended in supplemented SbM9 medium at 10^8 cells/ml. The cells were incubated under growing conditions for six hours to starve the cells of metabolites. After this time the cells were diluted to 10^7/ml. The mixture was incubated under growing conditions for 5 days. The cells were then washed twice with water and survivors plated on LB. Colonies that grew up in 3 days were replicated onto minimal medium to determine prospective auxotrophs. Requirements were determined by test streaking them on minimal plates supplemented with metabolite pools as described by Holliday (21). This technique gave 10^4-10^5 killing during the Carbenicillin treatment and 2% auxotrophs among the survivors.

Carbenicillin is effective at 2mg/ml., yet only ten fold killing takes place each day in minimal medium. This is surprising since the doubling time of Rm2011 in minimal medium is about 6 hours. One would then expect high levels of cell death within 24 hours. Even increased levels of Carbenicillin did not enhance killing.

Before enrichment the level of auxotrophs in the bacterial population is less than 0.1%, and afterwards the level is 2%. This shows that the enrichment scheme does work. However, the long incubation time and low

percentage of auxotrophs make this method more tedious than that described for E. coli (19). I have used this technique to isolate the auxotrophs Rm2012 - Rm3375 (II.C.I.).

Recently, another method has been described by Klapwijk, et al. (22) to isolate auxotrophs in Agrobactererium. In this method both Carbenicillin (500ug/ml) and lysozyme (100ug/ml) are used together to enrich for non-growing cells. I have found that PenG (1mg/ml) and lysozyme (100ug/ml) can be used in Rm2011. This method give 10^5 killing within 12 hours and 2% auxotrophs among the survivors. Penicillinase is added after incubation (2000 I.U./ml) to inactivate the PenG. I have used this technique to isolate mutants Rm3375-90 (II.C.I)

Multiply marked strains were isolated by repeated mutagenesis and enrichment.

II. H.

3. Tester strain Rm3330

A multiply marked strain of Rm2011 was isolated to be used in studies to establish genetic transfer. The strain was obtained with str3, and successively made resistant to spectinomysin (spc1, spontaneous), tryptophan requiring (trp33, EMS), and resistant to rifampin (rif1, spontaneous) to give strain Rm3330, genotype trp33, rif1, spc1, str3. The trp33 marker reverts less than 10^{-10} and the drug markers can be used in counterselection schemes.

II. I. PHAGE

1. Rhizobiophage

Phage that lyse Rm2011 were isolated as described by Parker and Allen (26). Soil isolates of about 10 grams were added to 50ml. of water and slowly shaken at 30°C overnight. The suspension was centrifuged to remove particulate material, chloroform was added to the supernatant, which was then plated on Rm2011 using the overlay method in SAY medium. Plaques that formed were purified by streaking on an indicator lawn of Rm2011 growing on SAY plates.

Transduction experiments using rhizobiophage were done by absorbing phage at moi of one to a log phase culture of Rm3330 grown in SAY medium. After incubation at 30°C for 2 hours the cells were diluted and plated on SbM9 minimal medium.

2. Enteric phage

Rm2011 was tested for sensitivity to enteric phage by spotting on a lawn of Rm2011 about 10^8 pfu of the phage listed in Table II-C-2. These phage included P22, P1vir, P1kc, T4, T5, T7, λ^+, λ_{cI60}, $\phi 80v_5$. There appeared to be clearing only by P1, yet even this phage produced no single plaques.

To isolate mutants of Rm2011 sensitive to enteric phage P1 and P22, I used the methods described by Goldberg, et al. (27). P1Cm and P22Tet were added at a moi of one to log phase clutures of Rm2011. Each culture was then plated on either chloramphenicol or tetracycline (depending on the phage) and incubated at 30°C. The Cm resistant colonies were purified and tested for growth, Cm^R, and phage production at 37°C.

II. J. ISOLATION OF R. MELILOTI FROM SOIL

Other rhizobia were isolated in an attempt to find naturally occurring fertile strains of R. meliloti. Bacteria were isolated from nodules of alfalfa plants that were growing on various farms. Excised nodules were first surface sterilized by dipping in ethanol for two minutes, followed by washing with water. They were then crushed and the contents streaked or diluted onto AA medium. Rhizobia can be recognized on this medium as white or clear mucoid colonies.

Several strains were isolated in this way from nodules, and several others from commercial sources. These naturally occurring rhizobia were used in liquid mating experiments with Rm3330 in an attempt to demonstrate genetic exchange. None of the strains isolated gave evidence for fertility.

These rhizobia were also screened for their ability to produce phage that would plaque on Rm2011. No phage were found.

II. K. MATING

A. LIQUID OR BROTH MATING

This type of mating procedure is routinely used to move RP4 into a new strain. It was also used in attempts to mate other plasmids into Rm2011.

Donors are grown to log phase in LB broth. Recipients are grown to saturation. Equal volumes of each parent are mixed and allowed to sit for 3 hours at 34°C. The mating mixture is then washed and re-suspended in the same volume of saline. Dilutions are then plated on appropriate media.

B. PLATE OR SURFACE MATING

This mating procedure is routinely used in RP4 promoted chromosome mobilization and linkage studies. This technique is the basis of the genetic mapping described in this thesis.

The donor which carries RP4 is grown to log phase in LB broth. The recipients are grown to saturation also in broth. One half ml. of both donor and recipient are mixed together and spread on a pre-warmed LB plate. After incubation overnight at 34°C, 10 ml. of saline is added to the resulting lawn and the resuspended bacteria are scraped into a centrifuge tube. The cells are washed twice and resuspended in 5 ml. saline, before being plated on selective media. Each parent alone is carried through the same procedure as a control. This technique allows mating to take place on a surface under nonselective conditions before recombinants are selected.

II. L. SELECTION

After mating, the mating mixture and controls are plated on selective medium. RP4 transfer was selected on LB agar with added tetracycline (10ug/ml). All auxotrophic markers selected for in mating experiments have a reversion frequency less than 0.1% of the recombinant frequency, except for pur-31 which gave revertants at about 1% of the recombinant frequency.

To counterselect donor bacteria, two different antibiotics are used when possible. In chromosome mobilization studies with RP4, counter-selection is done using a resistance marker as far away as possible from the auxotrophic marker being selected, (i.e. rif1 and trp33, pyr49 and nov59).

M. LINKAGE DATA

All linkage data are derived from RP4 plate matings. A single marker is first selected on plates lacking the required nutrient. Then 200 colonies are picked and streaked on appropriately supplemented plates, to score for other (i.e. unselected) markers. The cotransfer or linkage frequency for a given marker pair is defined as the fraction of selected colonies that have also inherited the unselected marker from the donor.

III. RESULTS

A. ISOLATION OF MUTANTS

In order to develop a genetic system in R. meliloti I first isolated a collection of auxotrophic and drug resistant mutants of Rm2011, as described in II. G.H. These isolates are listed in II.C.1. R. MELILOTI STRAINS. EMS was used to isolate all auxotrophic mutants except Rm2025-32, which were isolated following NTG mutagenesis.

Spontaneous mutants to most of the drugs to which Rm2011 is sensitive have been isolated. The drug, concentration in the selection, and strains carrying the resistance marker are listed in Table III-A.

TABLE III-A

DRUG	ABBREV.	CONC. (ug/ml)	STRAIN
Spectinomycin	spc	100	Rm3300-90
Rifampin	rif	50	Rm3330-90
Novobiocin	nov	50	Rm3357, Rm3359
Polymyxin B	pmx	2	Rm3388, Rm2101
Gentamicin	gen	50	Rm3391
Vancomycin	van	50	Rm2103
Erthromycin	ery	50	Rm3374, Rm2102
Naladixic Acid	nal	50	Rm3373
Chloramphenicol	crm	50	Rm1009
5-Fluorouracil	5fu	100	Rm2074
Ethionine	eth	100	Rm3301

Even after heavy mutagenesis, I was unsuccessful in isolating mutants resistant to NaN_3, Tet, or $HgCl_2$.

III. B. UNSUCCESSFUL ATTEMPTS AT GENETIC TRANSFER

I tried a number of other methods before succeeding with RP4. To do this, I used the tester strain Rm3330 trp33, rif1, spc1, str3, (see II.H.3).

1. Transformation

There have been many reports of transformation in Rhizobium. Balassa (1) first described transfer between three species using drug resistance markers. Lorkiewicz (5) and Dunican (23) have reported transformation in fast growing strains while Raina and Modii (4) have established techniques for the slow growing species R. japonicum.

I used DNA from the parental strain Rm2011, isolated as described by Marmur (24). I used the techniques described by Balassa, Dunican, and Raina and Modii in my attempts to transform Rm3330 to prototrophy. None of these experiments were successful.

Dunican has also described the transformation of the R-factor RP4 into R. trifolii. RP4 DNA was isolated using the Cleared Lysate procedure, and transformation by the technique previously described by Dunican (25), was used in an attempt to transform Rm2011 to Tet^R a drug resistance carried by RP4. RP4 can express Tet^R in Rm2011 following conjugation, and Rm2011 is very sensitive to Tet (2ug/ml). Nevertheless no transformants were detected among 10^9 cells.

I should point out that, although I followed published transformation procedures, I used Rm2011 rather than those particular strains used in those reports. However, other laboratories have also had difficulty reproducing published results, (J. Beringer, F. Cannon, pers. comm.).

There has been described a transformation technique in E. coli using $CaCl_2$ and cold shock (28). This procedure can be used to transform

plasmids, some of which carry Tet resistance into E. coli and Klebsiella pnemoniae. Chakrabarty (29) has used this method to transform RP4 into Pseudomonas.

J. Denarie's laboratory (pers. comm.) has carried out extensive studies with Rm2011 in order to demonstrate transformation. However, neither the classical rhizobia transformation procedures nor the cold shock, $CaCl_2$ technique have been successful in this strain.

I did not continue to work on transformation once the RP4 conjugation system began working.

2. Transduction

Generalized transduction has been described by Kowalski (8) who used the temperate phage L5, which he isolated after testing 30 different strains for lysogeny. L5 can transduce many markers at a frequency of 10^{-6}/pfu, but cotransduction has been demonstrated in only one instance (9). Sik and Orosz (7) have used the specialized transducing phage 16-3 to move a cys marker in R. meliloti 41. Rm2011 is not sensitive to either of these phage and since there were already laboratories using them I decided to look elsewhere for a transducing phage.

Phage that lyse Rm2011 were isolated as described in II and I (Phage). I have recently isolated three phage (HP1-3) from soil around alfalfa roots. None of these transduce Rm3330 to prototrophy. Other strains of rhizobia were screened for their ability to produce phage, by spotting chloroformed cultures on a lawn of Rm2011. No plaques were found. To test for transducing phage that were not lytic on Rm2011 derivatives, I grew these new R. meliloti strains with the tester strain Rm3330 for three days in mixed culture, and then tested for trp^+ on SbM9 rif, str plates where only Rm3330 could grow. I found no evidence for genetic exchange.

I then tried to isolate mutants of Rm2011 sensitive to the generalized transducing phages P1 and P22 of enteric bacteria. Goldberg et al, (27) have described how to select mutants sensitive to P1 among a population of bacteria not normally sensitive to this phage. P1Km clr100 carries a gene for kanamycin resistance. By adding this phage to a culture of bacteria and selecting for kanamycin resistance, one can isolate the rare P1-sensitive mutants that can be lysogenized by P1km phage. Since this phage is thermoinducible the lysogen can be induced by raising the temperature to 37^{o}.

Since Rm2011 is resistant to Km, I used another thermoinducible P1 derivative, namely P1Cm clr100, which carries resistance to Chloramphenicol instead. Infection of Rm2011 gave Cm^{R} colonies at 20 fold above background. However, at 37^{o}, where the phage is normally induced in E. coli, these colonies were still viable and Cm resistant. No phage release was detectable on lawns of either E. coli or Rm2011. Similar results were found by Goldberg et al. with Erwinia and P1km, and by Kaiser and Dworkin (30) with Myxococcus and P1Cm.

I tried to test if the Cm determinant had separated from the P1 phage genome and become integrated into the Rm2011 chromosome, thus giving rise to Cm^{R} Rm2011 with no phage produced. 200 of the colonies selected at 30^{o} on LBCm after the addition of P1Cm were tested for additional auxotrophic markers, which might have arisen due to P1CM insertion, by streaking on SbM9 Cm Plates at 30^{o} and 37^{o}. All of the colonies grew on these plates thus giving no evidence for "hopping" by the Cm resistance.

P22Tet is a derivative of the generalized phage P22 which carries a gene conferring resistance to Tetracycline. Infection of a mutagenized culture of Rm2011 with 10^{9} phage gave no Tet resistant colonies on LBTet plates.

3. Conjugation

Conjugation has the advantage in that large sections of the chromosome may be transferred. This allows the mapping of genes that are somewhat distant from one another. I will describe here my attempts to find sex factors in other strains of R. meliloti, and to introduce known fertility factors from other bacteria into Rm2011.

a. Natural sex factors

I isolated ten strains of R. meliloti from soil samples and alfalfa nodules. I also used strains sold commercially as inoculant, and strains from other laboratories. Each prospective donor strain was grown in SAY medium to log pahse, mixed with an equal volume of Rm3330 recipients grown in the same manner, and incubated for three days under growing conditions. At the end of this time the saturated cultures were washed and plated on SbM9 rif str to select for trp$^+$ Rm3330 derivatives. None were found.

b. Sex factors from other bacteria

The fertility factor F from E. coli is also fertile in Salmonella and Pasteurella, therefore I tried to mate it into Rm2011. Since there is no selection for F$^+$ cells, I used the episome F'ColVB trp isolated by Fredericq (31). I mated E. coli WD5017/F'ColVB trp with Rm3300 in liquid and plate matings as described in II.L. and selected for trp$^+$ on SbM9 str plates.

I found no trp$^+$ exconjugates of Rm3300 in 10^{10} cells. However in a different type of experiment described in Appendix II, I did isolate a recombinant of RP4 and F'ColVB trp that will mate into Rm3300. This shows that the selection was appropriate, since E. coli trp genes are expressed in R. meliloti.

I tried a similar experiment with F_{ts}*his* from *E. coli*. Using the *his* auxotroph Rm2049, I selected *his*$^+$ recombinants after liquid mating with *E. coli* RW11/ F_{ts}*his*. This selection was limited by the reversion of Rm2049 to prototrophy at 10^{-8}. I found no *his*$^+$ recombinants above the reversion frequency.

I also tried to mate into *R. meliloti* the fertility factor FP found in *Pseudomonas*. In *Pseusdomonas* FP increases resistance to $HgCl_2$ from 0.01mM to 0.1mM. Since this sex factor carries resistance to Hg^{++}, I could select those mutants of Rm2011 which would accept FP by selecting for increased resistance to $HgCl_2$. The minimal toxic level of $HgCl_2$ is o.1mM. Both liquid and plate matings were done with *Pseudomonas* PAO/FP and Rm3330, and selection was done on LB rif, str, o.1mM $HgCl_2$ plates. No colonies were found in 10^{10} cells. Therefore, either there is no mating, or FP cannot confer resistance to Hg^{++} upon Rm3330.

c. R-factors

Many R-factors promote chromosome mobiliation in various bacteria. F-type (R1) and I-type (r64) have been used in *E. coli* and *Salmonella*. The I-type (R144-drd3) was used to mobilize *Klebsiella* genes from *K. pnemoniae* to *E. coli* by Dixon and Postgate (32). P-type R-factors mobilize chromosomal genes in *Pseudomonas* (33). I will describe here my attempts to introduce R-factors of many different compatibility groups into Rm2011 from *E. coli*. Of all the R-factors tested, only RP1 and RP4 could mate into Rm2011.

E. coli carrying the R-factors are listed in III.B.3. Each was used as a donor in liquid mating with Rm2011 for 3 hours at 37^o. Selection was done on LB supplemented with str to counterselect the donor and with a drug to which Rm2011 is sensitive that is carried by the R-factor being tested.

Controls were done of each parent alone. The R-factors, their drug resistances, compatibility group, and the concentration of antibiotic used in the selection in LB str plates are listed in Table III-B-3. In order to be detected an R-factor would have to mate into Rm2011 at a frequency above 10^{-8}/ donor since 10^{8} donors were plated in each mating mixture.

TABLE III-B-3

R-factor	Compatibility	Resistances	Drug selected in LB strep plates	Conc. ug/ml	Freq.
J5-3 pro⁻ met⁻					
J5-3/R1	FII	ACSSu	C	50	$< 10^{-8}$
" /R1-16	FII o^c	"	C	50	"
" /R1-19	FII i^-	"	C	50	"
" /r386	FI	T	T	10	"
" /R124	FIV	T	T	10	"
" /R64	I	TS	T	10	"
" /R64-11	I drd	TS	T	10	"
" /N3	N	TSSu	T	10	"
" /S-a	W	CSKSu	C	50	"
" /R40a	C	ATK	T	10	"
" /R55	C	ACKGSu	C	50	"
" /R135	M	TSGSu	T	10	"
" /R6K	X	AS	Carb.	50	"
" /R71a	Y	ATCSSu	T	10	"
" /R402	T	ASSp	Carb.	50	"
" /RA1	A	Tsu	T	10	"
" /R387	K	SC	C	50	"
" /RP4	P	ATK	T	10	10^{-6}
E.coli C/R6886	P	ATK	T	10	$< 10^{-8}$
AB1932-1/R1822 or RP1	P	ATK	T	10	10^{-6}

Symbols: A-ampicillin, C-chloramphenicol, T-tetracycline, S-streptomycin, Sp-spectinomycin, Su-sulfathiazole, K-kanamycin

IV. RP4

A. INTRODUCTION

RP4 is a P-type drug resistance factor that confers resistance to Carbenicillin or Ampicillin, Tetracycline, and Kanamycin/Neomycin. It was found in a clinical isolate of Pseudomonas that had become resistant to Carbenicillin (34). Datta et al. (35) showed that RP4 will mate into most Gram-negative bacteria including Rhizobium. Stanisich and Holloway (33) used P-type R-factors to mobilize the Pseudomonas chromosome. RP1 and RP4 both were the only R-factors that would mate into Rm2011, and since no difference has been found between them, I chose RP4 for further study. I will describe here some of the properties of RP4 and its effect on Rm2011.

B. PROPERTIES

Rm2011 is very sensitive to Tet so this drug was used to select for the transfer of RP4 into Rm2011. Mating mixtures were plated on 10ug/ml Tet, colonies appearing were tested on LB plates supplemented with various drug concentrations by streaking for single colonies. Resistance is defined as the highest concentration of a drug at which single colonies can grow. The results are shown below.

	Amp	Tet	Kan	Neo
Rm2011	10	less 1	100	
Rm2011/RP4	20	10	150	50

Amp resistance is not expressed very well in Rm2011. Beringer reported similar results with RP4 in R. leguminosarum (36). Kanamycin is also not very useful for selection of RP4 since the level of Rm2011 is so high, and the increment of resistance added by RP4 is so small. For these

reasons transfer of RP4 was always selected using Tet 10ug/ml.

Olsen (37,38) has isolated two phage, PRD-1 (DNA) and PRR-1 (RNA), that plate only on bacteria carrying P-type R-factors. Neither phage lyses Rm2011/RP4, although in my hands both lysed the control E. coli J5-3/RP4.

Nodulation tests were carried out as described in II.F. RP4 does not alter the nodulation properties of Rm2011. This is essential for the future use of the RP4 mating system in studies of the genetics of nodulation.

Stability of RP4

RP4 is very stable in Rm2011. Even after repeated subculture in drug free LB no cured cells were found (fewer than 10^{-4}).

I tried to cure RP4 from Rm2011 using Acridine Orange, Ethidium Bromide, SDS, and Sarkosyl. Each agent was added to tubes of LB in two-fold dilutions from 200ug/ml to 1.5ug/ml. 10^3 cells of Rm2011/RP4 were added, and each tube was then incubated at 32^0 for 4 days. Cells from the tube with the highest concentration that allowed any visual growth were plated onto LB. The colonies that appeared were replicated onto LBTet plates to screen for curing. No Tet sensitive cells were found among 10^4 tested for each agent. Growth at 39^0, the high temperature limit for this strain, also gave no evidence for curing.

Datta, et al. (35) found that RP4 could not be cured in E. coli, consistent with my results in R. meliloti.

C. FREQUENCY OF TRANSFER

Liquid matings were done as described in II.L.1 and plated on LB agar supplemented as indicated. The results are shown below.

Donor	Recipient	Selection	Counter selection	Frequency/donor
E. coli J5-3/RP4	Rm2011	Tet 10ug/ml	Str	10^{-6}
Rm2011/RP4	*E. coli* EG47	Kan 25ug/ml	42^{o}	10^{-5}
Rm2011/RP4	Rm3330	Tet 10ug/ml	rif	10^{-4}

Controls with no RP4 in the donor gave no evidence of drug transfer. Frequency/ donor is defined as the number of selected markers per number of donor cells at the end of the mating.

Thus, RP4 can mate both into and out of Rm2011.

I found no evidence for chromosome mobilization in liquid matings as described in II.K.1. Rm2011/RP4 was mated overnight with Rm3330 *trp33*, *rif1*, *spc1*, with selection for Tet (RP4) transfer or *trp*$^{+}$ (chromosome mobilization). Tet transfer was 10^{-4}/ donor, consistent with earlier findings, but no *trp*$^{+}$ transfer was found (less than 10^{-9} *trp*$^{+}$ colonies/ donor).

In *E. coli* chromosomal markers are mobilized during matings at about 10^{-5} of the frequency of episome transfer. In *R. meliloti* liquid matings, RP4 is transferred at 10^{-4}/ donor. Therefore chromosomal mobilization at 10^{-5} of that frequency would give 10^{-9} recombinants per donor cell. This would put the level of gene transfer at the borderline of detectability. Attempts to improve RP4 transfer led me to develop the plate mating technique which increases transfer to 10^{-1}/ donor. This method and the resulting chromosome mobilization are described in the next section.

D. CHROMOSOMAL MOBILIZATION USING RP4

RP4 promoted chromosomal mobilization is the basis of the genetic system I have developed in R. meliloti. The plate mating technique is described in II.K.2. I describe here proof of mobilization and some properties of the system.

1. Proof of gene transfer

First, I mated Rm2011/RP4 with the tester strain Rm3330. Trp$^+$ recombinants were selected on SbM9 rif, spc plates and RP4 transfer was monitored by plating on LB rif Tet. Unmated parents were carried through as controls. In these condition, R-factor transfer was increased to 10^{-1}/ donor. In addition, trp$^+$ colonies were now found at 10^{-6}/ donor cell. Since Rm3330 reverts to trp$^+$ at less than 10^{-10}, and the donor cell Rm2011/RP4 mutates to simultaneous double resistance to rif and spc at less than 10^{-11}, the trp$^+$ colonies appeared to be due to genetic exchange. As a control, a mock mating of Rm2011 (carrying no RP4) with Rm3330 gave no trp$^+$ colonies. Therefore, the trp$^+$ colonies appearing in the cross Rm2011/RP4 X Rm3330 appear to represent mobilization of trp$^+$ by RP4.

Next, I was able to demonstrate the transfer of a nonselected marker. The donor Rm2074/RP4 ade14, 5fu74 was crossed with Rm3330 in a plate mating, with selection for trp$^+$ only. The trp$^+$ colonies were then tested for resistance to 5-fluorouracil. One hundred sixty of the 200 trp$^+$ colonies tested were also 5fu resistant. This transfer of a nonselected marker is additional proof that chromosomal mobilization has taken place.

2. Properties

These colonies appear to represent true haploid chromosomal recombinants by several criteria: a) when purified, colonies are stable and do not segregate parental types; b) unselected donor markers are found among

the selected colonies at characteristic and reproducible frequencies; c) the unselected markers may be auxotrophic and therefore presumably recessive. (see sample cross in next section).

The reason for the success of the plate mating technique has not been studied. Broth mating experiments using enough cells to detect 10^{-10} chromosome transfer were without success. It is possible that in plate matings the surface stabilizes pair formation since R-factor transfer itself is increased 1000 fold (10^{-4} to 10^{-1} per donor).

I find approximately the same frequency of recombination for all markers selected, (10^{-6} / donor). Since RP4 transfer is 10^{-1} / donor the recombination frequency is 10^{-5} per RP4 transferred. This is comparable to the frequency of mobilization in E. coli by other R-factors and F.

Fifty per cent of the recipients become R^+ after the overnight mating. However, when the recombinants are scored for the presence of RP4, 98% of them carry the R-factor. This has been found for every marker selected. The recombinants which are R^+ have the same donor ability as Rm2011/RP4. Recombinants which remain R^- have not yet been tested.

E. LINKAGE

Using the plate mating technique (II.K.2) I have crossed pairs of differently marked strains, selecting for one marker and then testing the recombinants for linkage of unselected markers (II.L.,M.). From the linkage frequencies I observed, I have constructed a linkage map of R. meliloti.

1. Procedure

RP4 was mated into each of the auxotrophic strains Rm2012 to Rm2032 from E. coli J5-3/RP4. These episomal donor strains were then crossed in

various pairs with the multiply marked recipient strains, Rm3339 to Rm3391. In each mating only one of the prototrophic markers from the donor was selected for. Two hundred recombinants were then scored for the presence of each unselected donor marker. The unselected donor markers were found among the selected colonies at characteristic and reproducible linkage frequencies.

A sample cross is performed as follows:

Rm2012/RP4 gly12 is plate mated with Rm3344 trp33, his39, pan44, rif1, spc1.

1) select for trp^+ on SbM9 gly, his pan, rif plates
2) select for pan^+ on SbM9 gly, his, trp, rif plates

Linkage is determined as follows:

1) trp^+ recombinants are scored for gly, his, pan
   out of 200 trp^+ --8gly^-, 45 his^+, 5 pan^+ (all pan^+ are gly^-)
2) pan^+ recombinants are scored for gly, his, trp
   out of 200 pan^+ -- 34 gly^-, 6 trp^+, 2 his^+

Since 200 recombinants of each selected marker were scored, linkage below 0.5% would not be detected. No linkage (less than 0.5%) was found between the following pairs of markers: pyr49--gly12, pur31--trp33, his39; ilv13--his39; leu53--rif1.

In the earliest experiements I had of course not even a rough idea of where markers were located on the map. Therefore, there was inevitably some distortion in cases where a counterselected marker was actually located near a selected marker, a fact which would emerge from several experiments done with different combinations. Ultimately I was able to arrange crosses choosing a counterselective marker that was located far from the selected marker. Thus, for example, when rif was used in matings in which pyr49

recombinants were selected, few pyr$^+$ colonies were found. It was only by using nov to counterselect the donor when selecting pyr$^+$ and scoring for rif that the high linkage between pyr49 and rif1 was found. Similarly, nov maps near trp33 and cannot be used as a counterselection when selecting for trp$^+$ recombinants.

All map positions shown in Fig IV-1 and IV-2 were determined in crosses where the counterselective marker mapped at least 120^0 on the circle from the selected marker.

2. Linkage Map

All the linkage results can be represented in a circular linkage map (Fig. IV-2). In Fig. IV-1 the circle has been broken arbitrarily between pur31 and cys11 so that the linkage frequencies from 1-5 crosses for each marker can be shown. The selected marker is at the tail of the arrow, and the head of the arrow points to the nonselected linked marker. The numbers on each tail are the cotransfer or linkage frequency in percent.

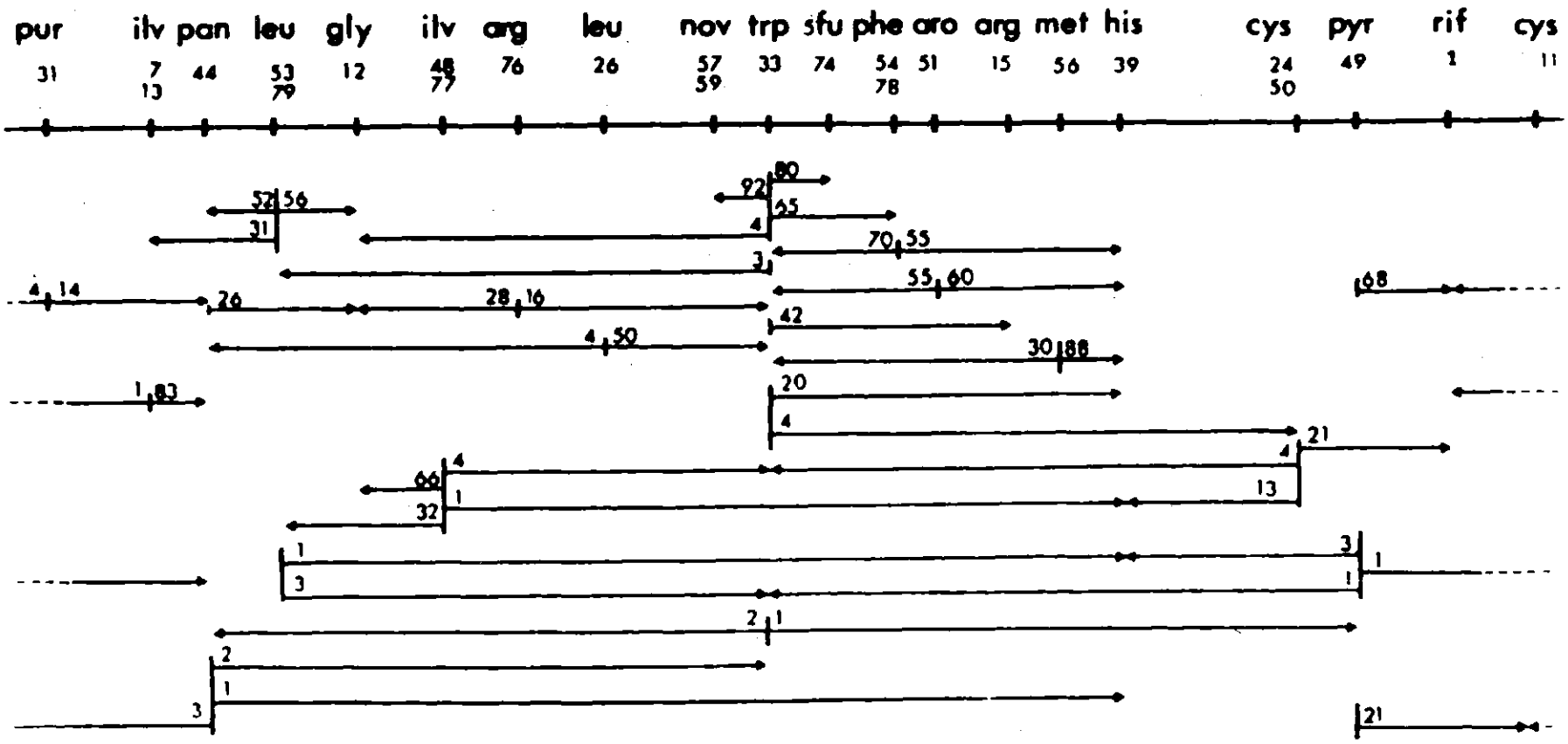
F. DISCUSSION

These results demonstrate transfer of genetic markers between R. meliloti strains.

The exconjugants with recombinant phenotype appear to be haploids resulting from true crossing-over, since they do not segregate, and since recessive markers may be introduced from the donor.

As expected, the empirical linkage map is one-dimensional. Further, selection of a pair of donor markers implies in most cases inheritance of a marker located between them on the map: for example in the cross arg15 X trp33, his39, simultaneous selection for trp$^+$ and his$^+$ gives recombinants of which 98% are arg$^-$. (see Fig. IV- -1)

FIG. IV-1 LINKAGE OR COTRANSFER FREQUENCIES USING RP4 OF *RHIZOBIUM MELILOTI*

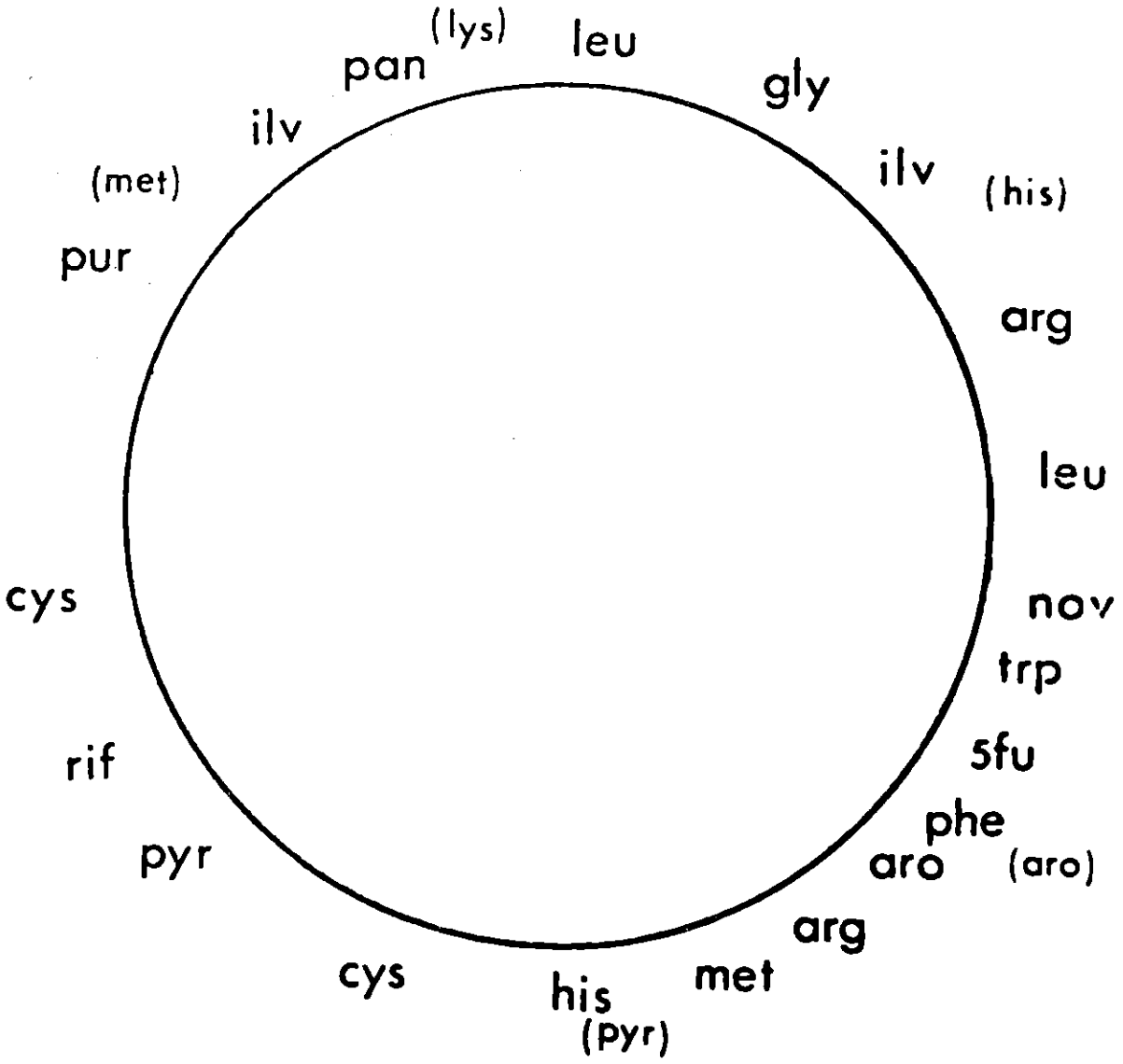


The selected marker is at the tail of the arrow,

and the head of the arrow points to the nonselected linked marker.

Fig. IV - 2

Linkage Map of Rhizobium meliloti



These results deal entirely with genetic linkage and hence prove nothing about the physical structure of the R. meliloti chromosome. Nevertheless, since the R. meliloti map is circular like those of enteric bacteria such as E. coli, it would not be surprising to find that the R. meliloti chromosome is circular too. Comparison of Fig. IV-E-1 with the map of E. coli (39) does have some rough similarities. For example the relative positions of leu53, trp33, his39, phe54 and aro51 seem to be close to that of E. coli. However, there are at least three markers that seem to be grouped at single loci in E. coli but at more than one in R. meilioti, (his, leu, and ilv).

Recently linkage of auxotrophic markers has been shown in R. japonicum using transformation techniques (6). Two linkage groups are found, one containing arg and ura, the other with ileu, val, leu. No linkage was found between the two groups. This may be similar to the map I have constructed for R. meliloti, (i.e. ilv13, leu53 and arg15, pyr80 which maps close to his39 on Fig. IV-E-2).

Beringer and Hopwood (16) have reported preliminary linkage data in R. leguminosarum, using the R factor, R68-45, to promote transfer. Three of the four markers which they have studied map in an order consistent with markers found on the R. meliloti map.

I have as yet little information about the mechanism of transfer. Since I found a wide continuum of linkage frequencies ranging from 0.5 to 92%, there is no reason to suppose that more than one mechanism is involved. Recombination frequencies for all markers are roughly the same and give no indication of a polarity of transfer. This is similar to F^+ X F^- matings in E. coli, where the origin and polarity of transfer that are characteristic of a single mating event are masked in the overall population by

the large number of such events with independent origins that take place simultaneously. This is likely to be the case here too.

This is unlike the RP4 promoted conjugation described in Acinetobacter (40) in which a gradient of recombination frequencies was found for different markers on the linkage map. However, I have isolated one R. meliloti derivative, that gives higher (100-10X) frequency of transfer of some markers, apparently with both an origin and polarity like an E. coli Hfr male, (see Appendix I). Thus at this early stage of understanding, the E. coli F system seems to be a useful model for RP4-promoted transfer in R. meliloti, although the situation in Acinetobacter might be different.

One disadvantage of this system is that almost all recombinants become R^+. Since I have been unable to cure Rm2011 of RP4, any strains that have had new markers crossed into them, can only be used as donors. This limits strain construction.

F-type Hfrs can be isolated selectively through the use of an F_{ts} derivative that is thermosensitive for episomal replication (41). I have isolated an analogous $RP4_{ts}$ and have attempted this kind of selection, (see Appendix IV). Other experiments which may elucidate the mechanisms of chromosome transfer, such as donor/ recipient ratio, time course of RP4 and chromosomal gene transfer, and the relationship of chromosomal mobilization to RP4 trnasfer itself have yet to be done.

This genetic system allows me to locate chromosomal markers anywhere on the linkage map of Rm2011. All of the 25 sites constitute a single linkage structure, and there is no evidence for any additional linkage groups. Therefore I expect to be able to link any new markers to markers

already mapped, and the linkage frequency should be no less than the frequency for the pair that is currently the most loosely linked, namely 3% (for cys11 and pan44).

I believe that this genetic system will greatly aid our study of Rhizobium genetics and physiology. But more importantly it now allows us to begin a genetic analysis of the Rhizobium-legume symbiosis. The most interesting properties of Rhizobium, namely nodulation and nitrogen fixation, cannot be easily selected in free-living bacteria. The mating system will allow the relevent genes to be manipulated by their linkage to more easily handled markers. This will allow us to construct strains with interesting combinations of mutations in the symbiotic process, a procedure which is vital to the genetic analysis of such a complex system.

When I began this research in 1972, there was no workable genetic system in a nodulating strain of Rhizobium. I chose R. meliloti strain Rm2011 for a number of reasons. First, this species nodulates alfalfa which is a small seeded legume and allows nodulation assays to be carried out in a laboratory of limited space. Second, Scherrer and Denarie had already begun working with this strain and were studying the effect of auxotrophic mutations on the nodulation process (42). Finally, and most important, R. meliloti grows rapidly and, unlike other rhizobia, on standard E. coli media.

The early parts of this thesis, which were unsuccessful attempts to show genetic exchange, were carried out when I was just learning microbiological techniques and genetics. For this reason I feel that some of these experiemnts warrent repeating. A transformation procedure has recently been described for Azotobacter (43). This technique might be effective with Rm2011. In my early attempts at phage isolation none were found, yet recently I succeeded in isolating three phage from soil samples. This project should be continued since the development of a transduction system would complement the RP4 mating genetics.

I spent a lot of time trying to find conditions where RP4 would promote chromosome mobilization. In fact, the hybrid episomes (see Appendix II) were isolated with the hope that more bacterial DNA on the episome would increase homology and therefore lead to chromosome mobilization. I found this to be true in E. coli, but could not demonstrate increased mobilization by the hybrid episomes in R. meliloti.

The plate mating technique is very simple and obvious in retrospect, since Holloway had used this method earlier in Pseudomonas (33). Both John Beringer in England and I hit upon this idea late in 1974. The reason for the success of the technique has as yet not been studied.

There is some concern over the use of a drug resistance factor in genetic studies. This is particularly true of RP4 because of its wide host range. Therefore I have autoclaved all cultures of bacteria carrying R-factors before discarding them, and petri plates with R^+ bacteria are incinerated. R. meliloti does have an important ecological niche. I have isolated many drug resistant mutants of Rm2011. To prevent the introduction of new genetic material carrying drug resistance information into the genetic pool of bacteria in the environment, I also sterilize cultures of Rm2011 before discarding.

The approach that I have taken to develop this genetic system may be of general importance. RP4 which was isolated originally from Pseudomonas is not native to R. meliloti. Therefore there should be nothing unique about R. meliloti which makes it amenable to genetic studies using RP4 promoted chromosome mobilization. For this reason it would seem that RP4 could be used in other bacteria. Because of its wide host range, RP4 may be used to develop genetic systems in a wide range of bacteria, many of which have interesting properties, yet no known genetic system.

Appendix I ISOLATION OF AN Hfr

Early mating experiments in E. coli were not easily interpretable until the isolation of an Hfr. Because of their higher frequency of recombination and directed transfer Hfrs make mapping much easier.

In an attempt to construct a strain carrying both pan$^-$ and gly$^-$ I accidently isolated an exconjugant that seems to have the properties of an Hfr. This strain gave 50-100 fold higher recombination frequencies for some markers, yet normal low levels for other markers.

STRAINS:

Rm2012/RP4 gly12, (ATK)

Rm3344 trp33, his39, pan44, spc1, rif1

Rm3357 " " leu53, " " nov57

Rm3359 " " pyr49, " " nov59

Rm3363 his39, pan44, gly12, rif1 (ATK)

CONSTRUCTION OF Hfr

My original goal was to construct a strain carrying both pan$^-$ and gly$^-$. To do this I crossed Rm2012/RP4 gly$^-$ with Rm3344 trp$^-$, his$^-$, pan$^-$, rif1, selected for trp$^+$ and scored for other markers. Three out of 200 were gly$^-$ and pan$^-$. One of these, Rm3363 his$^-$, pan$^-$, gly$^-$, rif1, (ATK) was chosen for further study.

Initially I crossed this new strain (Rm3363) with Rm3359 trp$^-$, his$^-$, pyr$^-$, nov59, rif1 and selected for either trp$^+$ or pyr$^+$ recombinants. Surprisingly, 100 fold higher (10^{-4}/donor) frequency of pyr$^+$ recombinants was found, yet a normal (10^{-6}/donor) level of trp$^+$ colonies appeared. This was the first evidence that Rm3363 may be an Hfr.

For this reason I did an interrupted plate mating with both Rm2012/RP4 and Rm3363 crossed with Rm3359. I selected for $\underline{trp}^+$ and $\underline{pyr}^+$ recombinants in order to determine the kinetics of chromosome mobilization. The time course of interrupted mating was done as follows:

Donor and recipient cultures were mixed together and one ml. of the mating mixture was spread onto each of six identical LB plates, (one for each time point). The plates were incubated and at each time point one was removed and the mating interrupted. This was done by adding 10 ml of Saline, scraping the lawn into a centrifuge tube, spinning, resuspending in 1ml of Saline, vortexing for 30 sec. and finally plating on selective media using F top agar. Controls of each parent alone and t=0 time point were done and showed no background $\underline{trp}^+$ or $\underline{pyr}^+$ colonies. This procedure was done at each time point. The results are shown in Fig. AI-1.

From this it appears that the pyr^+ marker from Rm3363 enters within 2 hours of the beginning of the mating process. However, the $\underline{trp}^+$ recombinants appear much later, at the same time and frequency as $\underline{trp}$ and $\underline{pyr}$ from Rm2012/RP4.

A three hour plate mating was then done between Rm3363 and recipients Rm3357 and Rm3359, in order to determine if $\underline{leu}^+$ also mates in early, and to study the linkage of $\underline{leu53}$ to $\underline{pan44}$ and $\underline{gly12}$. Under these conditions, $\underline{leu}^+$ recombinants were found at 10^{-5}/donor while the $\underline{trp}^+$ recombinants were below 10^{-6}. The $\underline{leu}^+$ were scored for $\underline{gly}^-$ and $\underline{pan}^-$. The results are shown in Table AI-1. Thus Rm3363 can mate both $\underline{pyr}^+$ and $\underline{leu}^+$ early in the mating process, and at a higher frequency than $\underline{trp}^+$.

TABLE AI-1 Hfr Linkage Data

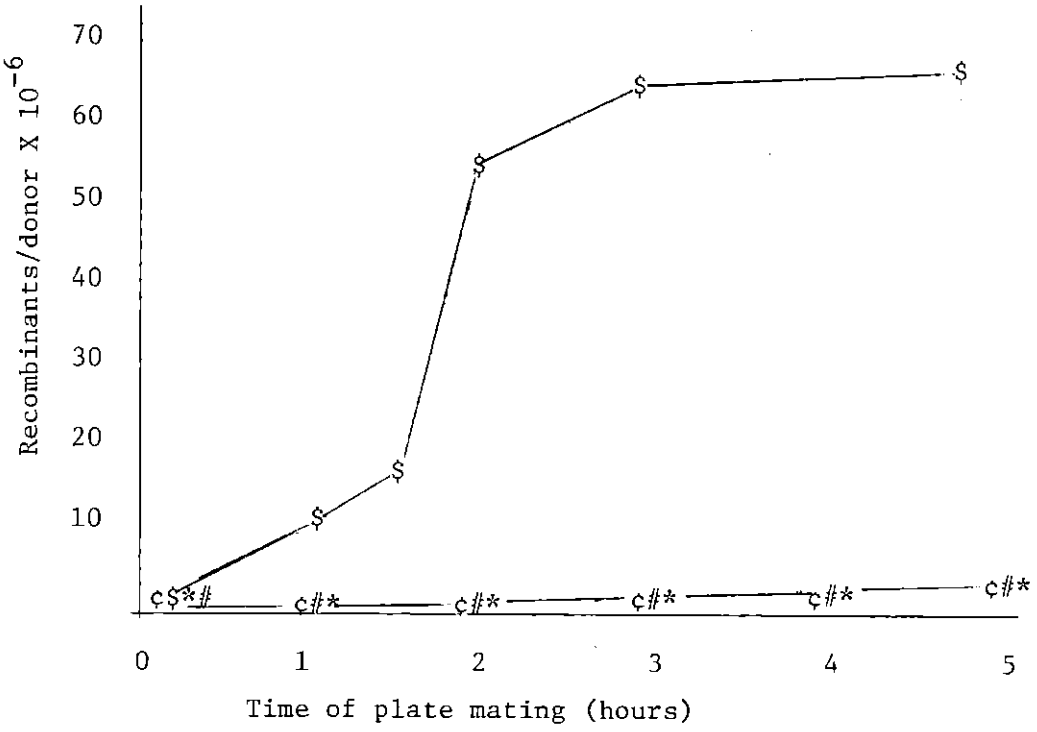
Donor	Recipient Selected Marker	pan$^-$	gly$^-$
Rm2012/RP4	leu53		56%
Rm3344/RP4	"	48%	
Rm3363 Hfr	"	86%	26%

Recombination Frequencies for Three Hour Plate Mating

Donor	Recipient	trp$^+$	leu$^+$	pyr$^+$
Rm3363	Rm3357	5×10^{-7}	5×10^{-5}	
	Rm3359	5×10^{-7}		10^{-4}

Figure AI-1 Time course of interrupted plate matings

Hfr Rm3363 X Rm3359 pyr$^+$ = $ trp$^+$ = ¢ Rm2012/RP4 X Rm3359 pyr$^+$ = # trp$^+$ = *



This is the first evidence of an Hfr in R. meliloti using RP4. As can be seen from the linkage data, true haploid recombinants are being formed since recessive alleles can be mated into the recipient (pan$^-$ and gly$^-$). What is more interesting is the difference in linkage of pan and gly to leu53 in low frequency as compared to Hfr matings, (see Table AI-1). The increase in pan and decrease in gly linkage in the Hfr cross is consistent with the model that transfer starts at pyr49 and proceeds clockwise through leu53. This would increase the relative frequency of pan over gly.

Due to the lack of suitably marked strains, further studies of the kinetics of transfer of markers between pyr49 and pan44 have not yet been done. Also, I do not know whether the RP4 is integrated, or is changed in this strain, Rm3363. The mating ability has not been tested in any of the strains that have been crossed with Rm3363 to determine what is responsible for this increased recombination frequency. However, this preliminary study does show that the RP4 mating system can yield what in F terminology is an Hfr.

Hybrid episomes that are recombinants of RP4 and F'ColVBtrp were isolated by a selective mating procedure. The episomes were then characterized to determine genes carried, type of pili formed, and mating ability. P1 transduction of RP4 and F' markers suggested that the recombinants isolated really represent hybrid episomes.

STRAINS:

WD5010 gal$^{=}$, [tonB-trp]$^{\nabla}$

WD5017/F'ColVBtrp " / F' att80, tonB, trp, cysB, ColVB

WD5160/F'ColVBtrp " / " , (ATK)

J5-3/RP4 pro^{-}, met^{-}

WD5148/pHM1 gal$^{=}$, [tonB-trp]$^{\nabla}$, /tonB, trp, ATK

WD5149/pHM2 " ,/ trp, AT

WD5150/pHM3 " ,/ trp, cysB, ATK

WD5154/pHM4 " ,/ att80, tonB, trp, cysB, ATK

WD5155/pHM5 " ,/ "

WD5157/pHM7 " ,/ tonB, trp, ColVB, ATK

X85 lac^{-}, trp^{-}, cysB, his^{-}, strA

X95 lac^{-}, trp^{-}, his^{-}, ura^{-}, (λ_{cI857})/1

KRO trp^{-}, recA, strA

EG47 gal$^{=}$, strA, r^{-}, m^{+}

Rm3300 trp33, spc1, str3

Rm3411/pHM1 " (tonB, trp, ATK)

Rm3413/pHM3 " (trp, cysB, ATK)

Rm3414/pHM4 " (att80, tonB, trp, cysB, ATK)

Rm3415/pHM5 " (")

Rm3417/pHM7 " (tonB, trp, ColVB, ATK)

MEDIA: All strains were routinely grown in LB. LBTet has 20ug/ml of Tetracycline, LBKm has 25ug/ml of Kanamycin, Str indicates the addition of 250ug/ml of Streptomycin. Minimal media GB_1M9 and SbM9 are described in II.B.

RESULTS

As I have shown in III.B.2., E. coli F'ColVBtrp mated with Rm3300 gave no trp^+ colonies above the detectable level of 10^{-10}. However if the donor E. coli carries RP4 as well, trp^+ colonies do appear.

The E. coli donor WD5160/F'ColVBtrp, RP4 used in the original selection was constructed by mating RP4 from J5-3/RP4 into WD5017/F'ColVBtrp in broth and selecting on GB_1M9 Tet, Km plates.

The original selection was done by plate mating WD5160/F'ColVBtrp,RP4 with Rm3300 trp33, spc1, rif1, str3, and selecting for trp^+ on SbM9 Str plates. Trp^+ colonies appeared at 10^{-10}/donor. The experiment was then repeated twice, each time giving the same low frequency of trp^+ colonies. These Rm3300 trp^+ derivatives,Rm3411-17,were then mated back into E. coli WD5010 in broth with selection for trp^+ colonies on GB_1OM at 42^o which appeared at 10^{-5}/donor. Several of these E. coli, WD5148-57, were chosen for further analysis.

When WD5148-57 were mated back into Rm3300 using the broth technique, trp^+ colonies were found at 10^{-6}/ donor. This is the same frequency that RP4 mates from E. coli to R. meliloti (see IV.C). This suggested that the original selection for trp^+ at 10^{-10}/ donor resulted in trp^+ episomes that have the same mating properties as RP4. The episomes which were now in the WD5010 background were then characterized as described below. They were found to have RP4, P-type pili, drug resistances, and varying genes from F'ColVBtrp, which inclued one or more of att80, tonB, trp, cysB and ColVB.

The E. coli strains and episomal genotypes, along with the Rm3300 trp$^+$ derivatives from which they came are listed below.

	R. meliloti	E. coli
Parent	Rm3300	WD5160/F'ColVBtrp, RP4
Exp. 1	Rm3411	WD5148/pHM1
		WD5149/pHM2
Exp. 2	Rm3413	WD5150/pHM3
Exp. 3	Rm3414	WD5154/pHM4
	Rm3415	WD5155/pHM5
	Rm3417	WD5157/pHM7

CHARACTERIZATION OF EPISOMES

cysB: mate with X85, select for trp$^+$, score for cysB$^+$

tonB: cross streak with $\phi 80_{v5}$, tonB$^+$ are sensitive, tonB$^-$ resistant

att80: cross streak with λ_c+h80, test single colony isolates for immunity to λ_{cI60}, mate with X95 selecting for trp$^+$ at 30°, then test trp$^+$ for ability to grow at 42. (see App. III)

Pili formation:

F-type pili- cross streack with MS2, sensitivity indicates F pili production

P-type pili- cross streak with PRD-1, sensitivity indicates P pili

Mating ability: donors replicated onto lawn of recipients spread onto selective plates

Preliminary studies of chromosome mobilization in E. coli and R. meliloti were done. The hybrid episomes show no difference in ability to promote chromosome mobilization in Rm2011 as compared to RP4. However

in E. coli the hybrid episomes promoted chromosome mobilization in broth mating, while RP4 does not.

In order to show that these episomes are in fact recombinants and not just RP4 and F'ColVBtrp coexisting in the same cell, I tested for cotransduction using P1. Markers on the same episome should be cotransducible, while those on separate episomes should not. Using the methods described by Rosner (44), I grew P1Cm lysates of WD5160, WD5148-50. These lysates were then used to transduce WD5010 at moi of 0.1 to trp^+. The transductants were then scored for other markers found on the episomes from which the P1 lysate came. The results are shown below in Table AII-2.

TABLE AII-2

P1 lysate	trp	tonB	A	T	K	P pili	Ability to mate out
WD5160	100	52	0	0	0	0	0
WD5148/pHM1	100	96	3	3	96	3	3
WD5149/pHM2	100	ND	48	47	ND	41	41
WD5150/pHM3	100	ND	96	96	96	96	96

These results show that drug resistance is linked to trp in the recombinant episomes but not in the parent WD5160/F'ColVBtrp, RP4. A and T resistances are closely linked to P-type pili production and mating ability, yet Km resistance is not. The linkage between trp and ATK is 96% for pHM3. I found that this episome can be transduced into KRO trp^-, recA, strA, to yield trp^+ colonies that retain the ability to mate out. None of the other episomes tested could be transduced into a recA strain. This plus the high linkage between trp and ATK suggests that pHM3 is small enough to fit entirely into P1.

These episomes should make it possible to move the E. coli genes on them into other bacteria. For example, the results described here show that E. coli trp is expressed in R. meliloti. Since two of the episomes carry att80, there is also the possibility of introducing into R. meliloti the coliphage ϕ80 or specialized transducing derivatives of this phage. Appendix III describes one such experiment, the introduction of λh80 into Rm2011.

Appendix III INTRODUCTION OF COLIPHAGE λh80 INTO R. MELILOTI

The hybrid episome pHM5 (App. II) was used as a vector to introduce a coliphage λh80 into R. meliloti. The phage λ_{cI857}h80 could not be induced while in Rm2011.

STRAINS:

Rm3300 trp33, spc1, str3

WD5155/pHM5 gal$^{=}$, [tonB-trp]∇, (att80, tonB, trp, cysB, ATK)

X95 lac^{-}, trp^{-}, his^{-}, ura^{-}, strA, (λ_{cI857}) /1

EG47 gal^{-}, strA, r^{-}, m^{+}

Rm3427/pHM5 trp33, spc1, str3, /pHM5 (λ_{c}+h80)

Rm3428/pHM5 " /pHM5 (λ_{cI857}h80)

PHAGE:

λ_{c}+h80

λ_{cI857}h80

λ_{cI60}

METHODS:

All matings were done using broth mating technique (II.K.1). Lysogens were detected by stabbing from a single colony into a lawn of sensitive indicator, EG47.

RESULTS

WD5155/pHM5 was first lysogenized with λ_{c}+h80 by cross streaking the strain with λ_{c}+h80 phage. Single colony isolates were then tested for immunity to λ_{cI60} to make sure they were lysogens. At this point it was not possible to know whether the phage was on the episome or chromosomally integrated. To determine this, six isolates were broth mated with X95,

with selection for growth at 42°, due to complementation of the λ_{cI857} repressor by λc^{+}h80. All six showed evidence that the phage had integrated into the episome.

One of thse WD5155/pHM5 (λ_c+h80) was mated with Rm3300 and *trp*$^{+}$ colonies were selected on SbM9 plates. The frequency of *trp*$^{+}$ colonies was 10^{-4}/ donor with no decrease due to the presence of the phage on the episome. These *trp*$^{+}$ colonies were purified and tested for lysogeny by stabbing into a lawn of EG47. Clearing due to phage production was found in 24/25 colonies tested. One of the Rm3427, was used in further studies to show that λ_c+h80 was actually carried into *R. meliloti*.

If the phage was present on the episome within Rm3427, then it should be possible to mate this episome into X95 (λ_{cI857})/1 selecting for *trp*$^{+}$ at low temperature, 34°. Complementation of the λ_{cI857} by pHM5 (λ_c+h80) should now allow these bacteria to grow at 42°. This was done, first by broth mating Rm3427 with X95, selecting for *trp*$^{+}$ at 34°, then scoring for the ability to grow at 42. Five out of 50 *trp*$^{+}$ derivatives of X95 were now able to grow at 42. Lysates from one of these showed both phage present. Thus is appears that Rm3427 was indeed a lysogen of λ_c+h80.

Induction of λh80

Since there was clearing around the stab of Rm3427 into a lawn of EG47, one would expect that phage were being produced by *R. meliloti*. To test this, the thermoinducible phage, λ_{cI857}h80 was mated into Rm3300 using pHM5 as described above. *Trp*$^{+}$ colonies selected on SbM9 str plates also gave clearing when stabbed into a lawn of indicator. One of these *trp*$^{+}$ colonies Rm3428 (λ_{cI857}h80) was used in an attempt to produce phage from *R. meliloti*.

When cultures of Rm3427 and Rm3428 were chloroformed and spotted on lawns of EG47, no phage were found. Even after temperature induction of Rm3428 no phage could be found in the culture. However, when either of these strains were plated on the indicator as viable bacteria, phage plaques were observed. In fact, every viable bacterium was an infective center of phage production. Thus, it seems that R. meliloti cannot produce the phage but it can mate it out into E. coli where it is induced.

Further experiments were done in an attempt to induce phage from Rm3428. Even after growth at 37^{o} overnight there was no phage in the culture nor any killing of the R. meliloti. Heavy mutagenesis with NTG also failed to produce any mutants of bacteria or phage that were expressed in culture.

Even though R. meliloti cannot produce ϕ80, the episome pHM5 can be used as a vehicle to introduce those genes that have been isolated on ϕ80 specialized transducing particles. Since pHM5 has the same broad host range as RP4, one would expect to be able to mate these phage into a wide range of bacteria.

Appendix IV ISOLATION OF $RP4_{ts}$

F-type Hfrs can be isolated selectively in E. coli through the use of an F_{ts} that is temperature sensitive for episomal replication (41). This procedure involves the use of F_{ts}lac, which replicates autonomously at low but not at high temperatures. If this episome is present in a strain that carries a point mutation in the chromosomal lac gene (i.e. lac^-/F_{ts}lac), the cells are lac^+ at low temperature. When shifted to the higher temperature the F_{ts}lac stops replicating and is diluted out of the population. However, colonies that remain lac^+ can be isolated at the high temperature. In these colonies the F_{ts}lac has integrated into the lac region by homology, where it is passively replicated.

If the lac region of the chromosome is deleted, this region of homology is no longer present. F_{ts}lac can still integrate into other regions of the chromosome, although at a lower frequency, (10^{-4}). The cells in both situations become Hfr males which transfer their chromosome from an origin at the site of integration.

By analogy I have isolated a temperature sensitive derivative of RP4 and have attempted this type of selection in order to isolate an Hfr of RP4 in R. meliloti. Even though the selection for an Hfr has not yet worked, I will describe here how I isolated RP4-4, the temperature sensitive mutant.

GOAL: To obtain a mutant of RP4 that is unable to replicate at 37^o, yet replicates as wild type at 30^o

RATIONALE:

The screening for a temperature sensitive RP4 was based on the idea that cells carrying $RP4_{ts}$ would be unable to grow at the nonpermissive

temperature on drug plates. This property can be used to recognize those cells carrying temperature sensitive mutants of RP4 by the following procedure. LBTet, Km plates inoculated with cells carrying mutagenized RP4 are grown at the low, permissive, temperature until microcolonies appear, then the plates are shifted to the high, nonpermissive, temperature and incubated for 36 hours. During this second incubation, colonies carrying wild type RP4 will continue to grow and form large colonies, whereas any cells with $RP4_{ts}$ should remain as microcolonies and thus be detectable. Any mutants found can then be tested for temperature sensitivity at 37^{o} since that is the maximum temperature at which R. meliloti can grow well.

J5-3/RP4 was mutagenized heavily with NTG as described in II.G. using 100ug/ml for 30 min. at 37^{o}. Survival was 10% and 1% of these were lac^{-} as determined by plating on Lac McConkey agar. Following mutagenesis the cells were incubated for 3 hours at 28^{o} in LB, then mated with EG47 for 3 hours at 28^{o} in order to cross the mutagenized RP4 episomes into a non-mutagenized background. The mating mixture was then plated on LBTet, Km, Strep to select for EG47/RP4 colonies, at a dilution that would give about 200 colonies per plate. The plates were then incubated at 28^{o} until microcolonies appeared (18 hours), then shifted to 42^{o} for 36 hours. At the end of this time, the plates were screened for microcolonies.

Of 5000 colonies screened, 23 microcolonies were found. These were then tested for growth on LBTet and LBKm agar. Five were unable to grow on either drug plate at 42^{o}, but only one of these had the desired phenotype of not being able to grow on drug plates at 37^{o}. This mutant, RP4-4, was then tested for curing.

Curing was tested by streaking for single colonies on LB at various temperatures (28, 30, 34, 37). Progeny from the outside of a colony

appearing at each of these temperatures were then tested for Amp, Tet, and Km resistance at 28°. EG47/RP4-4 streaked at 37° on LB lost all three drug resistances, while at 28 or 30° it did not. From this, it appeared that RP4-4 was cured at 37, indicating that it is temperature sensitive for replication at 37°. Controls of EG47/RP4 showed no evidence for curing or temperature sensitivity.

This episome, RP4-4, was then mated into Rm2012 by broth mating at 28° for 3 hours, followed by selection on SbM9 gly, Tet plates at 28° to yield Rm2012/RP4-4. This strain was then tested for growth on LBTet and LBKm (150ug/ml) at 30 and 37°, and showed the same temperature sensitive phenotype as EG47/RP4-4. Rm2012/RP4 showed no temperature sensitivity.

SELECTION OF Hfrs

Selection for persistence of one RP4-4 drug resistance at high temperature was used in an attempt to isolate Hfrs. This was done by selecting for Tet resistance at 37°. A culture of Rm2012/RP4-4 was plated on LB and LBTet at 30 and 37°, and incubated for 4 days. The number of colonies that grew at 37 on LBTet was 10^{-3}-10^{-4} of the number that grew at 30 on LB or LBTet. However, this same low number appeared on the LB plates at 37° as well, with a background of microcolonies equal in number to the colonies that grew at 30°. Thus is appeared that RP4-4 inhibits growth of Rm2012 at 37° on LB, even in the absence of the drug.

Two hundred of the large colonies that grew on LBTet at 37° were purified under the same conditions. These were then tested for new auxotrophic requirements by streaking on SbM9 gly Tet plates at 37°. A new requirement would indicate integration of RP4-4 into a detectable gene. All 200 grew on these minimal plates.

Since there was no evidence for integration, five of these large colonies were then tested for their mating ability, along with controls of Rm2012/RP4 and Rm2012/RP4-4. All seven donors were plate mated at 28° with Rm3357 *trp*⁻, *his*⁻, *leu*⁻, as described in II.K.2. No difference in recombination frequency was found for either *trp* or *leu*. R factor transfer was also identical in all cases. Rm 3357 recipients, each carrying one of the R factors tested, were then screened for temperature sensitivity of drug resistance. The Tet and Km resistance of the R factors which came from the large colonies isolated at 37° were now temperature sensitive in Rm3357, just as RP4-4. Thus the suppression of the temperature sensitive phenotype was probably a mutation on the Rm2012 chromosome.

I have as yet no evidence that Hfrs can be generated by this technique. However, I intend to screen more temperature resistant colonies of Rm2012/RP4-4 for new markers and also to isolate more $RP4_{ts}$ derivatives.

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