JOURNAL OF BACTERIOLOGY, July 1984, p. 125–129
0021-9193/84/070125-05$02.00/0

Vol. 159, No. 1

Generalized Transduction in *Rhizobium meliloti*

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Received 22 December 1983/Accepted 3 March 1984

Generalized transduction of *Rhizobium meliloti* 1021 was carried out by bacteriophage N3. Genetic markers on the chromosome and the pSym megaplasmid were transduced, along with markers on several IncP plasmids. Cotransduction between transposon Tn*5* insertions and integrated recombinant plasmid markers permitted correlation of cotransductional frequencies and known physical distances. Bacteriophage N3 was capable of infecting several commonly used strains of *R. meliloti*.

Rhizobium meliloti forms a symbiotic and specific association with a host plant, alfalfa, resulting in the formation of nitrogen-fixing root nodules. Although recombinant DNA analysis of some symbiotic loci has progressed rapidly (1, 4), there remains a need for improved methods of strain construction and fine-structure mapping for the genetic analysis of *Rhizobium* spp. (4, 15).

Chromosomal linkage maps, derived by R-factor-mediated conjugation, have been constructed for several *Rhizobium* species, including *R. meliloti* (7, 13, 17, 23). Several bacteriophages capable of generalized transduction have been reported for *R. meliloti* (8, 31), but these phages do not infect the widely used strains derived from SU47, such as strain 1021. In this paper, we describe a bacteriophage, N3, capable of generalized transduction in strain 1021. The size of the viral genomic DNA was estimated after restriction endonuclease digestion. Phage N3 is capable of infecting several other commonly used strains of *R. meliloti*. This phage appears similar to the transducing phage described in the accompanying paper by Finan et al. (12).

(Some of these results were presented at the Ninth Annual North American *Rhizobium* Conference, Cornell University, Ithaca, N.Y., June 1983.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. Table 1 lists bacterial strains, plasmids, and phages used. *R. meliloti* and *Escherichia coli* were maintained on standard LB and M9 (24) or TY (3) media. Bacterial matings were carried out by the method of Meade and Signer (23). Phages MP1 through MP4 were obtained by screening samples of soil obtained from an alfalfa field (Coachella Valley, Calif.), using the techniques of Raleigh and Signer (26). Phages were propagated and titered on LB medium supplemented with 2.5 mM $CaCl_2$ (LB plus Ca^{2+}) as described by Lesley (18).

Initial screening for transduction of *R. meliloti* was carried out by the UV-irradiated lysate technique as described by Buchanan-Wollaston (6). Subsequent N3 transductions were conducted by using a citrate wash method to prevent progeny phage killing of recipient bacteria. A sample of phage lysate grown on donor bacteria was added (multiplicity of infection of less than 1) to a small volume of recipient bacteria grown in liquid LB plus Ca^{2+}. After an adsorption period of 30 min at 30°C, 1 ml of LB plus 10 mM sodium citrate was added, and the transduction mixture was incubated at 30°C for several hours to allow phenotypic expression of transduced genes. The cells were concentrated by centrifugation, washed three times in 5 mM sodium citrate, suspended in a small volume of 10 mM $MgSO_4$, and plated on selective media. Adsorption of phage N3 was assayed as described by Raleigh and Signer (26).

Construction of plasmid integrant strains. Plasmids were conjugated into *R. meliloti* with pRK2013 as a helper plasmid (9) by the method of Meade and Signer (23) and Ditta et al. (10). Cells were plated out on selective medium to determine the proportion of putative integrant cells. Like Kondorosi et al. (16), we found that the level of pBR325-borne tetracycline resistance is lower in *R. meliloti* containing integrated plasmids than in *E. coli* containing the free plasmid (2 μg/ml and 20 μg/ml, respectively).

Symbiotic assays. Alfalfa seeds (*Medicago sativa* cv. AS-13R; Ferry Morse Company) were sterilized, germinated, and inoculated with bacteria for nodulation tests as described by Meade et al. (22). Plants were maintained in a growth chamber (25°C, 16 h of light at 78 microeinsteins m^{-2} s^{-1}). Nodulation phenotype was scored at 5 weeks.

DNA techniques. *E. coli* plasmids were isolated by small-scale alkaline lysis as described by Maniatis et al. (20). Phage and plasmid DNA were cleaved by restriction endonucleases (Bethesda Research Laboratories and New England Bio-Labs) and analyzed by agarose gel electrophoresis, filter transfer, and hybridization with nick-translated radioactive DNA probes as described by Maniatis et al. (20).

Phage N3 DNA was isolated as follows. N3 was added to an exponentially growing 50-ml culture of strain 1021 in LB plus Ca^{2+} at a multiplicity of infection of 1, allowed to adsorb at 30°C for 30 min, and then added to 1 liter of medium prewarmed to 30°C. The flask was shaken for 8 h at 30°C, when lysis became evident. Cell debris was removed by centrifugation, and the supernatant was recentrifuged at 6,000 × *g* for 26 h. The pellets were gently suspended overnight in 10 ml of 10 mM Tris-hydrochloride (pH 8)–10 mM $MgSO_4$. The suspended phage were adjusted to 20 mM Na_2EDTA (pH 8)–0.5% sodium dodecyl sulfate–0.5 mg of autodigested protease (Sigma Chemical Co.) per ml. The preparation was incubated at 37°C for 60 min and extracted twice each with buffer-saturated phenol, phenol-chloroform (1:1), and chloroform. DNA was precipitated twice with ethanol and dissolved in 10 mM Tris-hydrochloride (pH 8)–1 mM Na_2EDTA.

RESULTS AND DISCUSSION

Transduction by phage N3. *R. meliloti* 1021 is insensitive to phage 11 and phage $DF2_{br}$, which are capable of generalized transduction in other strains of this species. $DF2_{br}$ failed to

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TABLE 1. List of bacterial strains, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics	Source or reference
Rhizobium meliloti		
1021	Sm^r derivative of SU47	22
2011	Sm^r derivative of SU47	23
2012	2011 *gly-12*	H. Meade
2013	2011 *ilv-7*	H. Meade
2026	2011 *leu-26*	H. Meade
3342	2011 *trp-33 his-39 cys-42 spc-1 rif-1*	H. Meade
3390	2011 *trp33 his-39 pan-44 spc-1 rif-1 nov-59*	H. Meade
1023	1021 *met-23*::Tn*5*	22
1099	1021 *trp-99*::Tn*5*	Long, unpublished
1491	1021 *nifH1491*::Tn*5*	28
1128	1021 *fix-28*::Tn*5*	Long, unpublished
1126	1021 *nod-26*::Mu::Tn*5*	19,22
S160	1021 *nod-160*::Tn*5*	Jacobs and Long, submitted
S161	1021 *nod-161*::Tn*5*	Jacobs and Long, submitted
S172	1021 *nod-172*::Tn*5*	Jacobs and Long, submitted
S212	1021 *lys-212*::Tn*5*	This paper
S217	1021 *phe-217*::Tn*5*	This paper
S218	1021 *dct-218*::Tn*5*	This paper
S219	1021 *his-219*::Tn*5*	This paper
S222	1021 *arg-222*::Tn*5*	This paper
S223	1021 *dct-223*::Tn*5*	This paper
S226	1021 *arg-226*::Tn*5*	This paper
1021::J2	1021::pRmJ2 cointegrate	This paper
1021::J3	1021::pRmJ3 cointegrate	This paper
F51S	Sm^r derivative of 102F51	W. Leps
1400	Rough derivative of Rm41	S. Long
L5-30	Wild type	M. Duncan
F34	Wild type (also 102F34)	G. Ditta
11CS	Sm^r derivative of 11C	S. Long
Escherichia coli		
LE392	*supE supF hsdR met trpR lac*	11
Phages		
MP1-MP4	Virulent, infects 1021	This paper
7a and N3	Virulent, infects 1021	18
$DF2_{br}$	Broad host range variant of virulent transducing phage DF2; 1021 is resistant	J. Casadesús
ϕ11	Temperate transducing phage for strain 41 and derivatives; 1021 is resistant	31
Plasmids		
pRK290::Tn*5*	Tc^r Nm^r	M. Orbach, unpublished
pPH1JI	Gm^r Sp^r	J. Beringer
R68.45	Tc^r Nm^r Ap^r	J. Beringer
GY1	R68.45 *cys-42 att*$^{16\text{-}3}$	14
pRK2013	Nm^r *mob* RP4, ColE1	10
pBR325	Tc^r Ap^r Cm^r	5
pRmJ2	pBR325 + 3.8-kb *Eco*RI fragment near the 1021 nodulation region	Jacobs and Long, submitted
pRmJ3	pBR325 + 3.5-kb *Eco*RI fragment near the 1021 nodulation region	Jacobs and Long, submitted

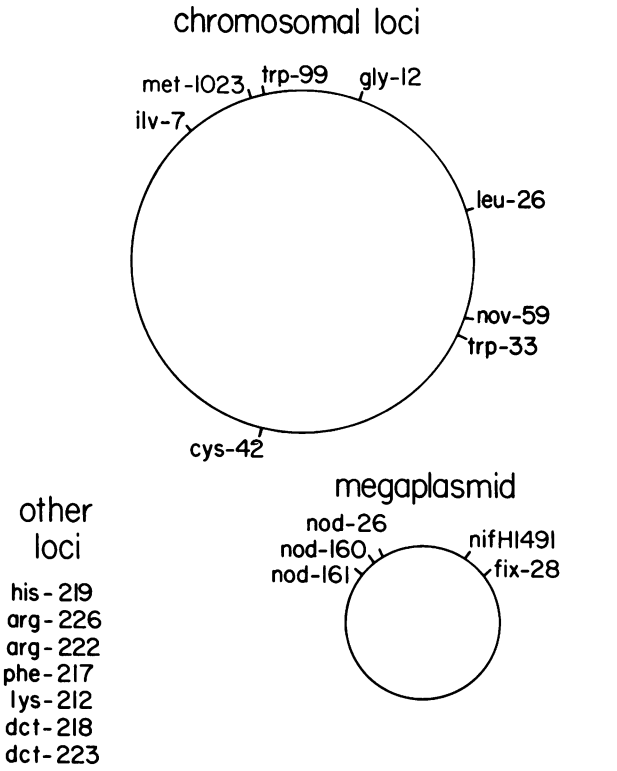

FIG. 1. Selected chromosomal and megaplasmid genetic loci of *R. meliloti* 1021 transduced by phage N3. Genetic markers with known map locations are shown accordingly. Other transduced genetic loci listed are presently unmapped.

bind appreciably to strain 1021 in adsorption assays (data not shown). Since neither transducing phage infected strain 1021, we screened a number of phages infectious on this strain for transducing ability, including four local soil isolates (MP1 through MP4), and two phages, 7a and N3, from the Lesley (18) typing collection. Lysates of each phage grown on prototrophic strain 1021 were used to transduce recipient strain Rm3390, a *trp-33* derivative of strain 1021 which reverts at a low frequency (ca. 10^{-9} per cell) (23). Samples of each lysate were UV-irradiated for various times to determine the optimum irradiation level, and transductions were carried out selecting for tryptophan prototrophy. Several of the phages displayed transductional activity. Of these, N3 showed the highest frequency, ca. 5×10^{-6} Trp$^+$ transductants per PFU, after 2,200 ergs of UV irradiation per mm^2.

When phage N3 was used to transduce from strain 1023 (*met-1023*::Tn*5*) to recipient strain 1021, Nm^r transductants arose at a frequency of 5.6×10^{-6} per PFU. Of 100 Nm^r transductants, all were Met$^-$; this confirms the generalized transducing activity of N3, since the *met-1023*::Tn*5* locus is distant from the *trp-33* locus on the Rm1021 genetic map (22). Various chromosomal and megaplasmid genetic markers (Fig. 1), as well as several plasmids, have been transduced with roughly similar frequencies into *R. meliloti* 1021 (Table 2).

Transposons inserted in or adjacent to symbiotic genes located on the large resident symbiotic plasmid (2, 27) of *R. meliloti* were also transducted by N3. Strains 1126 and 160 are Nod$^-$ mutants marked by Mu::Tn*5* and Tn*5*, respective-

TABLE 2. Generalized transduction of *R. meliloti* 1021 by bacteriophage N3

Strain or plasmid	Relevant marker	Phenotype		Frequency of transduction[b]
		Selected	Unselected[a]	
R. meliloti				
2012	*gly-12*	Gly^+		2.6×10^{-6}
2013	*ilv-7*	Ilv^+		3.8×10^{-6}
2026	*leu-26*	Leu^+		4.2×10^{-6}
3342	*cys-42*	Cys^+		3.7×10^{-6}
3390	*trp-33*	Trp^+		3.6×10^{-6}
1023	*met-23*::Tn*5*	Nm^r	Met^-	5.6×10^{-6}
1099	*trp-99*::Tn*5*	Nm^r	Trp^-	8.0×10^{-6}
1491	*nifH1491*::Tn*5*	Nm^r		2.8×10^{-6}
1128	*fix-28*::Tn*5*	Nm^r		2.0×10^{-6}
1126	*nod-26*::(Mu)Tn*5*	Nm^r	Nod^-	2.1×10^{-7}
TJ160	*nod-160*::Tn*5*	Nm^r	Nod^-	4.5×10^{-6}
TJ161	*nod-161*::Tn*5*	Nm^r		4.6×10^{-6}
TJ172	*nod-172*::Tn*5*	Nm^r		1.3×10^{-6}
SR212	*lys-212*::Tn*5*	Nm^r	Lys^-	8.0×10^{-7}
SR217	*phe-217*::Tn*5*	Nm^r	Phe^-	3.4×10^{-6}
SR218	*dct-218*::Tn*5*	Nm^r	Dct^-	1.1×10^{-6}
SR219	*his-219*::Tn*5*	Nm^r	His^-	1.7×10^{-6}
SR222	*arg-222*::Tn*5*	Nm^r	Arg^-	1.9×10^{-6}
SR223	*dct-223*::Tn*5*	Nm^r	Dct^-	5.1×10^{-6}
SR226	*arg-226*::Tn*5*	Nm^r	Arg^-	2.8×10^{-6}
Plasmids				
pRK290::Tn*5* (26 kb)		Nm^r		3.4×10^{-7}
pPH1J1 (47 kb)		Gm^r		1.7×10^{-7}
R68.45 (56 kb)		Nm^r		1.2×10^{-6}
GY1 (82 kb)		Nm^r		1.2×10^{-6}

[a] Transductions were carried out by the citrate wash technique as described in the text. Tn*5* mutants were selected for drug resistance and screened for the appropriate phenotype. At least 100 Nm^r transductants were screened except for nodulation mutants, with 30 transductants screened.

[b] Frequencies of transduction of selected markers were measured per PFU in each transduction and are averages of several experiments.

ly (19; T. W. Jacobs and S. R. Long, submitted for publication); 30 Nm^r transductants generated from each donor strain were shown to be Nod^- in plant tests (Table 2).

IncP plasmids ranging in size from 27 to 82 kilobases (kb) were also transduced with frequencies similar to chromosomal or megaplasmid markers (Table 2). After transduction, the plasmids were conjugated into *E. coli*, and all drug resistances remained intact. The highly efficient transduction of plasmids in *R. meliloti* is particularly useful for plasmid exclusion experiments (28); plasmid donor lysates are stable and may be used in many experiments, and transduction eliminates the need for counterselection markers in each recipient.

Cotransductional analysis. Of the markers known on the *R. meliloti* chromosomal genetic map, few are located close to one another as determined by conjugational mapping. Novobiocin resistance (*nov-59*) and *trp-33* are conjugationally linked with a frequency of 92% (23). When strain 3390 was transduced to Trp^+ by strain 1021, 94 of 192 Trp^+ transductants carried the linked nov^s allele, yielding a cotransduction frequency of 49%.

Using cloned segments of pSym megaplasmid DNA, we constructed strains with selectable genetic markers at defined positions to analyze cotransduction of closely linked sites. Plasmid pBR325 is incapable of replicating in *Rhizobium* spp. but can be efficiently mobilized by the *trans*-acting plasmid pRK2013 (9). We introduced pBR325 recombinant plasmids bearing fragments of megaplasmid DNA into *R. meliloti* and selected Tc^r to obtain cointegration of the pBR325-based clone at a known site (Fig. 2). We determined the arrangement of the integrated fragments by digestion of genomic DNA of each cointegrant with *Hin*dIII, for which there is a site in pBR325 but not in either inserted fragment. Electrophoresis, filter transfer, and hybridization of such digests with radioactively labeled insert (J2 or J3) DNA indicated that each cointegrant bore a duplication of the insert band, with pBR325 between the two copies (data not shown). A similar strain construction technique has been used in *E. coli* (32), *Myxococcus xanthus* (25, 30), and *R. meliloti* (16).

N3 lysates were prepared on strains 1128, 1491, 161, and 172, carrying Tn*5* (Nm^r) insertions, and on integrant strains (Tc^r) 1021::pRmJ2 and 1021::pRmJ3. Each was used to transduce into strains carrying the other marker. The unselected drug resistance marker was screened by replica plating. Loss of the original recipient marker showed cotransduction of the two loci (Table 3). As expected, cotransductional frequency increased with decreasing distance between the markers (Table 3 and Fig. 2). Analysis of the data with the Wu equation which relates marker distance to cotransduction frequency (34) yielded an average value for phage length of 187 kb.

Characteristics of phage N3. When plated on *R. meliloti* 1021 on LB plus Ca^{2+} medium and incubated overnight at 30°C, phage N3 gave rise to clear plaques (diameter, 1 to 2 mm), which approximately doubled in size upon extended incubation. Twenty stable N3-resistant clones recovered after infection were found to be nonlysogenic by stab tests. Transductants resulting from infection by N3 were sensitive to the phage. Transduction frequency decreased greatly at a multiplicity of infection of greater than 0.1 (data not shown). These characteristics suggest that N3 is virulent rather than lysogenic (31).

N3 requires Ca^{2+} for efficient infection (18). Adsorption of

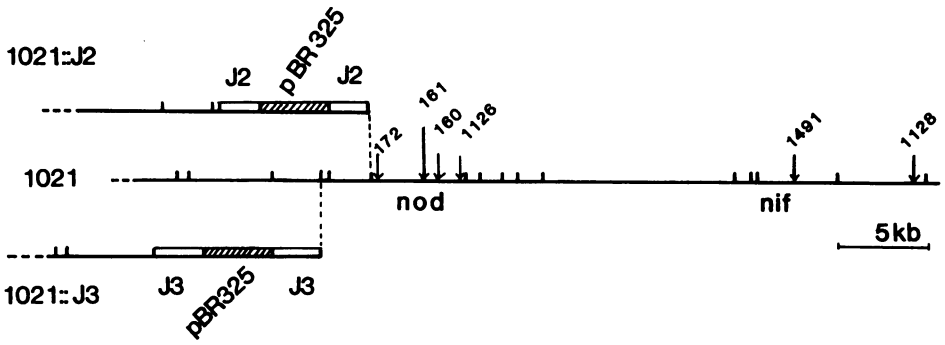

FIG. 2. Map of transposons and cointegrate structures in the *R. meliloti* 1021 nodulation-nitrogen fixation region. Tn*5* insertions are represented as arrows (19, 28; Jacobs and Long, submitted). J2 and J3 refer to *Eco*RI fragments of the megaplasmid cloned into pBR325 to yield pRmJ2 and pRmJ3 (Jacobs and Long, submitted). Integration events involving these plasmids as described in the text are represented as 1021::J2 and 1021::J3. Open boxes signify the duplicated *Eco*RI fragments, and plasmid pBR325 sequences are shown as hatched boxes.

N3 to strain 1021 in LB, LB plus Ca^{2+}, and LB plus Ca^{2+} plus 10 mM sodium citrate was assayed as described above. Neither LB alone nor LB plus Ca^{2+} plus 10 mM citrate facilitated efficient adsorption of the phage to strain 1021, whereas adsorption in LB plus Ca^{2+} was quite rapid (data not shown).

Purified phage DNA was digested with 15 enzymes recognizing 6-base-pair sequences, all of which cleave host genomic DNA. Of these enzymes, *Acc*I, *Bcl*I, *Bgl*I, *Cla*I, *Eco*RI, *Hin*dIII, and *Mlu*I cleaved N3 DNA, and *Bam*HI, *Bgl*II, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I, and *Xho*I did not. Many phages possess modified DNA (21), which renders them resistant to restriction endonuclease action (33). The probability of the large N3 genome lacking restriction sites for eight endonucleases is small, implying that some type of modification of bacteriophage DNA is occurring.

Some modification-sensitive restriction enzymes do cleave phage DNA. For example, *Msp*I and *Hpa*II, which are sensitive to methylation of different cytosine residues within their recognition sequence (5′ CCGG 3′), both digest N3 DNA to small-molecular-weight fragments with apparently identical patterns (data not shown). We have also found that *Sma*I and *Sal*I do not cleave N3 DNA (recognition sequences 5′ CCCGGG 3′ and 5′ GTCGAC 3′, respectively), whereas the enzymes *Hpa*II-*Msp*I and *Taq*I, which cleave at 5′ CCGG 3′ and 5′ TCGA 3′, do digest the viral DNA to small fragments. On a purely random basis, 1 of 16 *Hpa*II-*Msp*I or *Taq*I sites should also be an *Sma*I or *Sal*I site. It appears that N3 mediates sequence-specific modification of DNA, rendering the DNA insensitive to some restriction endonucleases but sensitive to others.

The restriction enzyme *Hae*III, which recognizes a 4-base-pair sequence (5′ GGCC 3′), digested N3 DNA to yield 21 fragments. Analysis of cloned N3 DNA indicates that this is due to a lack of sites rather than to modification (data not shown). The *Hae*III restriction fragments were sized and indicated the length of N3 DNA to be at least 190 kb, which was consistent with our cotransduction data.

Host range of N3. The host range of N3 includes *R. meliloti* 1400, L5-30, F34, and F51S, but not 11CS. Infection of strain F51S by N3 gives rise to a productive phage life cycle and titers comparable to those found with Rm1021 as host. Transduction was demonstrated by using pRK290::Tn*5* as the transduced marker at a frequency of ca. 5×10^{-6} Tc^r Nm^r colonies per PFU. We also observed an apparent restriction or modification system in strain 1021 that was not

TABLE 3. Cotransduction in *R. meliloti* with Tn*5* and integrated plasmid markers

Donor	Recipient	Marker distance (kb)[a]	Phenotype		Frequency of cotransduction[d]
			Selected[b]	Unselected[c]	
1128	1021::J3	46	Nm^r (140)	Tc^r (57)	0.41
1021::J3	1128	46	Tc^r (38)	Nm^s (18)	0.47
1491	1021::J3	41	Nm^r (127)	Tc^s (62)	0.49
1021::J3	1491	41	Tc^r (121)	Nm^s (60)	0.50
1491	1021::J2	37	Nm^r (130)	Tc^s (74)	0.57
1021::J2	1491	37	Tc^r (127)	Nm^s (69)	0.54
TJ161	1021::J3	12	Nm^r (158)	Tc^s (126)	0.80
1021::J3	TJ161	12	Tc^r (174)	Nm^s (143)	0.82
TJ161	1021::J2	8	Nm^r (131)	Tc^s (110)	0.84
1021::J2	TJ161	8	Tc^r (196)	Nm^s (176)	0.90
TJ172	1021::J3	8	Nm^r (164)	Tc^s (145)	0.88
1021::J3	TJ172	8	Tc^r (154)	Nm^s (137)	0.89
TJ172	1021::J2	4	Nm^r (180)	Tc^s (163)	0.91
1021::J2	TJ172	4	Tc^r (164)	Nm^s (153)	0.93

[a] Distances were estimated from known cosmid mapping data in Long et al. (19) and Ruvkun and Ausubel (28).
[b] Number of drug resistant transductants screened for the unselected marker is given in parentheses.
[c] Number of screened colonies sensitive to the antibiotic tested is given in parentheses.
[d] Cotransductional frequencies were determined by dividing the number of transductants sensitive to the unselected drug resistance marker by the total number of transductants as described in the text.

present in strain F51S; the efficiency of plating of strain 1021 lysates plated on F51S was ca. 1, and the efficiency of plating of F51S-grown N3 on strain 1021 was 2.5×10^{-2}. This value resembles that found with two temperate phages infecting *Rhizobium leguminosarum* and *Rhizobium trifolii* (29). Although spot testing indicated that N3 infected strains 1400, L5-30, and F34, we did not recover progeny phage from these strains. However, we did observe transduction of selectable markers from strain 1021 into strains 1400 and L5-30 with low frequency.

The generalized transduction of *R. meliloti* by bacteriophage N3 will be useful in genetic and molecular studies, including fine-structure genetic mapping, strain constructions, and enhanced mutagenesis of specific regions of the megaplasmid or chromosome. Such a transductional system will further advance the genetics of this bacterium.

ACKNOWLEDGMENTS

We thank S. Lesley for the generous gift of his phage typing collection, T. Sik for φ11, J. Casadesús for $DF2_{br}$, T. W. Jacobs for plasmids and *R. meliloti* strains, and J. T. Mulligan and R. F. Fisher for helpful discussions. We are also grateful to T. Finan and E. Signer for communicating results before publication and to the reviewers of this manuscript for their detailed suggestions.

This work was supported by the Department of Energy (contract no. DE-AT03-82ER 12084 to S.R.L.) and a National Institutes of Health Cell and Molecular Biology Training Grant to Stanford University (GM 07276-09) traineeship to M.O.M.

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