JOURNAL OF BACTERIOLOGY, Apr. 1987, p. 1403–1409
0021-9193/87/041403-07$02.00/0

Vol. 169, No. 4

Rhizobium meliloti Insertion Element ISRm2 and Its Use for Identification of the *fixX* Gene

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Received 30 July 1986/Accepted 17 December 1986

Two of the three plasmids of the wild-type *Rhizobium meliloti* 41 (pRme41*a* and pRme41*c*) carry a copy of ISRm2, a 2.7-kilobase-long transposable element. ISRm2 is terminated by 22-base-pair (bp) inverted repeat sequences, exhibiting some homology to the inverted repeats of elements generating 9-bp target sequence duplication. Transposition of ISRm2 results in a duplication of 8 bp in length, rather rare among transposable elements. DNA sequences homologous to an internal fragment of ISRm2 were found in several *Rhizobium* species. Transposition of ISRm2 into fixation and nodulation genes located on the symbiotic plasmid pRme41*b* was detected at a high frequency. Exact locations of two copies of ISRm2 which transposed into the *nod-nif* region on the megaplasmid were determined. In one case, integration into the protein-coding region of the *hsnD* gene that determines a host specificity function of nodulation occurred. In the other mutant, ISRm2 was localized upstream of *nifA*, where a short open reading frame coding for a new *fix* gene (*fixX*) was identified. The product of *fixX* is a ferredoxin carrying a characteristic cluster of cysteine residues. On the basis of the observation that the arrangement of the ISRm2 copies is identical in the free-living wild-type cells and in nitrogen-fixing nodules, we concluded that the involvement of ISRm2 transposition in the development of nitrogen-fixing symbiosis is unlikely.

Symbiotic nitrogen-fixing *Rhizobium* species are able to specifically interact with leguminous plants. Both bacterial and plant genes are involved in the formation of root nodules on the host plants, where atmospheric nitrogen is converted to ammonia.

In *Rhizobium meliloti*, genes coding for nitrogen fixation enzymes (*nif* genes) and for other functions of symbiotic nitrogen fixation (*fix* genes) are localized on a megaplasmid (3, 32). Bacterial genes determining nodule initiation (*nod* genes) and host specificity of nodulation (*hsn* genes) are also carried by the megaplasmid in the vicinity of the *nif* genes (24, 26).

Detailed studies on the genes participating in symbiotic nitrogen fixation unexpectedly led to the initial discovery of transposable elements in *Rhizobium* spp. (33). In both transposition properties and structural organization, the rhizobial insertion (IS) sequences resemble the procaryotic IS elements from other species (9). In *R. lupini*, the unusual instability of the plasmid RP4 is due to insertions of ISR1, a 1.15-kilobase (kb)-long transposable element of the *Rhizobium* genome, into three regions of RP4 (31). Another insertion sequence (ISRm1), 1.4 kb in size, was characterized by its ability to insert at a frequency of 10^{-2} to 10^{-3} into three *Eco*RI fragments carrying *nif* genes in *R. meliloti* 1021 (33). Repeated sequences RSRjα and RSRjβ clustered around the *nif* region were found in the slow-growing *Bradyrhizobium japonicum* (21). RSRjα is structurally similar to the IS elements, but its transposition has not been detected.

In this report, we describe a 2.7-kb-long transposable element of *R. meliloti* 41 designated as ISRm2. We show that ISRm2 carries 22-base-pair (bp)-long inverted repeats at its termini, and an 8-bp target sequence duplication is induced by its insertion. In the wild-type *R. meliloti* 41, the two copies of ISRm2 are localized on the indigenous plasmids pRme41*a* and pRme41*c*. The transposition of ISRm2 into *fix* and *nod* genes carried by the megaplasmid pRme41*b* was detected. As one of the targets of ISRm2, a new *fix* gene, *fixX*, which codes for a ferredoxin, was identified between *fixC* and *nifA*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The strains, plasmids, and bacteriophages used in this study are listed in Table 1.

Media and growth conditions. *R. meliloti* and other *Rhizobium* species were cultured as described previously (24). *Escherichia coli* strains were grown in LB complete medium or in M9 minimal medium (27).

Transposon Tn*5* mutagenesis. Random Tn*5* mutagenesis with plasmids pSUP1011 and pJB4JI was carried out as described by Simon et al. (35), and Beringer et al. (4), respectively.

Detection of large plasmids on an agarose gel. The in-gel lysis method of Eckhardt (13) as modified by Forrai et al. (15) was used to separate plasmids and chromosomal DNA.

Plant tests. The symbiotic properties of *R. meliloti* derivatives were tested on the host plant alfalfa (*Medicago sativa* L.) as described previously (23).

DNA isolation. Plasmid DNA was prepared by the method of Birnboim and Doly (5) and purified by two steps of CsCl-ethidium bromide density gradient centrifugation. Total DNA from bacteria was prepared by the method of Dhaese et al. (11) and from root nodules by the method of Varsanyi-Breiner et al. (39). M13 phage derivatives used for sequencing were propagated and purified by the Amersham protocol (1).

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Vector	Characteristics of strain or cloned fragments of plasmids and phages	Reference or source
R. meliloti 41			
AK631		Nod$^+$ Fix$^+$ wild type, compact colony morphology	This laboratory
ZB138		Nod$^-$ Fix$^-$ derivative of AK631 carrying deletion on pRme41*b*	3
ZB129		Nod$^-$ Nif$^-$ derivative of AK631, with a deletion on pRme41*b*, lacking pRme41*a*	24
ZB308		Nod$^-$ derivative of AK631 obtained after Tn*5* mutagenesis	24
AK1485, AK1486, AK1487, AK1488, AK1489, AK1490, AK1491, AK1492, AK1493, and AK1494		Fix$^-$ derivatives of AK631 obtained after random Tn*5* mutagenesis	This work
R. meliloti 1021		Nod$^+$ Fix$^+$ wild type	F. Ausubel
R. leguminosarum			
JB897		*phe trp str* derivative of *R. leguminosarum* 300	J. Beringer
1015			J. Beringer
248			J. Beringer
R. trifolii 24K/56			Z. Lorkiewicz
A. tumefaciens Ach5			M. van Montagu
E. coli HB101		*hsdS hsdM pro leu thi gal lacY recA str*	6
pACYC184	Tc^r Cm^r		10
pAK9	pBR322	12.8-kb *Bam*HI fragment of *R. meliloti* 41 *nif* region	2
pID2	pACYC184	4.8-kb *Eco*RI fragment of *R. meliloti* 41 with IS element	This work
pSK10	pACYC184	6.8-kb *Eco*RI fragment of *R. meliloti* 41 with IS element	This work
pHM5	pJC307	Carrying Tn*5*	28
pEK5022	pJB8	49-kb fragment of Rme41*b*	24
pEK10	pRK290	6.8-kb *Eco*RI fragment of *R. meliloti* 41	24
pSK12	M13mp18	*Hin*dIII-*Bam*HI fragment of *R. meliloti* 41	This work
pSK15	M13mp19	*Hin*dIII-*Bam*HI fragment of *R. meliloti* 41	This work
pID21	M13mp8	*Hin*dIII-*Pst*I fragment of pID2	This work
pID23	M13mp8	*Hin*dIII-*Pst*I fragment of pID2	This work
pSK40	M13mp19	*Hin*dIII-*Bam*HI fragment of pID2	This work
pSK31	M13mp8	*Mlu*I-*Xho*I fragment of pSK10	This work
pSK22	M13mp19	*Hin*dIII-*Xho*I fragment of pSK10	This work

Cloning procedures. For the cloning of IS-containing fragments, total DNA of the mutant strains (ZB308 and AK1488) was digested with *Eco*RI and separated on a 0.7% agarose gel. Restriction fragments were isolated and ligated with T4 DNA ligase into *Eco*RI-digested and dephosphorylated pACYC184 vector as described by Maniatis et al. (27). *E. coli* was transformed by the method of Lederberg and Cohen (25). The cloning of DNA fragments for sequencing into M13 vectors digested with appropriate enzymes was carried out by the Amersham protocol (1). *E. coli* JM101 was used for transfection. Digestions with restriction enzymes were performed by the protocols of New England BioLabs. T4 DNA ligase was kindly provided by K. Burg. The Klenow fragment of *E. coli* DNA polymerase I was prepared in this institute. Alkaline phosphatase was a product of Boehringer GmbH.

Hybridization. Radioactively labeled probes were prepared by nick translation as described by Maniatis et al. (27). DNA was hybridized with the probes either in-gel (15) or after Southern transfer onto nitrocellulose filters (36). The conditions for hybridization were described previously (3, 15). Clones containing recombinant plasmids were screened by colony hybridization (16).

DNA sequencing. For the determination of the DNA sequence, the dideoxy chain termination method of Sanger et al. (34) was used. Preparation of the template, sequencing reactions, and polyacrylamide gel electrophoresis were performed as specified by Amersham Corp. (1). The primers were kindly provided by A. Simoncsits.

RESULTS

Detection of an IS element in *R. meliloti* after random Tn*5* mutagenesis. To isolate symbiotic mutants of *R. meliloti* 41 affected at different stages of nitrogen fixation, AK631 was mutagenized with transposon Tn*5* by using the suicide plasmids pJB4JI (4) and pSUP1011 (35). Both Nod$^-$ and Fix$^-$ derivatives were selected in plant tests. By studying the linkage between Tn*5* and the mutant phenotype, we observed that in certain cases, the symbiotic defect was not due to the Tn*5* insertion but instead to the transposition of an indigenous IS element.

In one typical experiment, after introduction of pSUP1011, 1,200 independent Km^r *R. meliloti* derivatives were selected and tested on alfalfa for symbiotic ability. Ten transconjugants exhibited the Fix$^-$ phenotype (Table 2).

Various symbiotic functions of *R. meliloti* are encoded by the megaplasmid pRme41*b* (3, 24, 32). To identify Tn*5*

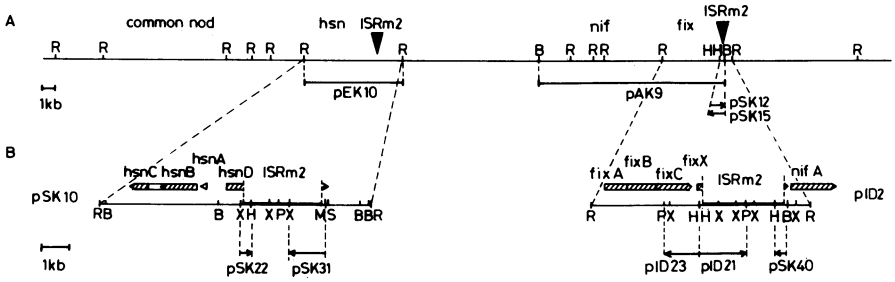

FIG. 1. (A) *Eco*RI restriction map of the *nod-nif* region of *R. meliloti* 41. In addition to the *Eco*RI sites (R), two *Hin*dIII (H) and two *Bam*HI (B) sites are shown. Plasmids pEK10 and pAK9 were used as hybridization probes to identify *Eco*RI fragments carrying ISRm2. (B) Restriction maps of the two cloned *Eco*RI fragments. A heavy line demonstrates the IS elements within the fragments. Above the physical maps of pSK10 and pID2, the protein-coding regions are labeled with hatched arrows: *hsnABC*, *hsnD*, and *fixABCX*, *nifA*, respectively. Arrows below the physical maps represent restriction fragments cloned into M13 derivatives for DNA sequencing. Abbreviations: P, *Pst*I, X, *Xho*I; M, *Mlu*I.

insertions in this megaplasmid, chromosomal DNAs and plasmids of the Fix$^-$ mutants were separated by gel electrophoresis (3, 13) and then hybridized directly in the gel with a Tn*5* probe (pJC307::Tn*5* plasmid; data not shown). The Tn*5* probe hybridized to the megaplasmid in 7 of 10 Fix$^-$ mutants and to the chromosome in the 3 remaining mutants (Table 2).

A relatively short region of the symbiotic megaplasmid carries nodulation and fixation functions (24). To determine whether the new Tn*5* insertions were in this region, *Eco*RI fragments of total DNAs from wild-type and mutant bacteria were separated by gel electrophoresis and hybridized with cloned *nif-fix* region DNA (pEK5022 cosmid clone [24]). Changes in the hybridizing fragment pattern attributable to insertion of the 5.8-kb Tn*5* element were screened for. Results are summarized in Table 2. One of the mutants, AK1491, showed the pattern anticipated for Tn*5* insertion into a *fix* gene: a fragment of the wild type was replaced by a 5.8-kb-larger fragment (which hybridized when the filter was probed with labeled Tn*5*). However, in other mutants, the increase in size did not correspond to the molecular weight of Tn*5*. For example, in AK1488, a 4.8-kb fragment was replaced by a 7.5-kb fragment, and the Tn*5* probe hybridized to a 15-kb fragment. On the basis of these results, we concluded that the insertion of a putative transposable element of unknown origin had occurred in several mutants.

Cloning of DNA fragments carrying IS sequences. To characterize the inserted DNA, *Eco*RI fragments containing the transposable element were cloned from the Fix$^-$ mutant AK1488 and from the Nod$^-$ mutant ZB308. In AK1488, the IS sequence was localized in a 4.8-kb *Eco*RI fragment (Fig. 1) carrying *fix* genes and *nifA* (8). In the Nod$^-$ mutant ZB308, the element was inserted into a 6.8-kb *Eco*RI fragment (Fig. 1) coding for the host specificity genes of nodulation (24).

*Eco*RI fragments of the total DNAs from both mutants were separated by gel electrophoresis. Fragments carrying the IS elements were isolated from the gel and ligated into *Eco*RI-digested pACYC184. The *Bam*HI fragment of plasmid pAK9 (2), partially overlapping the 4.8-kb *Eco*RI fragment (Fig. 1), was labeled and used as a probe to identify clones carrying the IS from AK1488; labeled pEK10 containing the wild-type 6.8-kb *Eco*RI fragment (Fig. 1) was used for screening the recombinant clones from ZB308.

Two recombinant plasmids were selected for further studies: pID2, carrying the 4.8-kb *Eco*RI fragment with the IS sequence, and pSK10, containing the 6.8-kb *Eco*RI fragment with the inserted sequence. The positions of the inserts within the *Eco*RI fragments were mapped by using restriction enzymes (Fig. 1). The inserted DNA does not carry restriction sites for *Eco*RI, *Bam*HI, *Kpn*I, *Bgl*II, *Sma*I, *Xba*I, or *Bst*EII. Three *Xho*I sites were found in the IS copy in pID2 and, interestingly, only two sites in the other copy in pSK10. *Hin*dIII and *Pst*I have one restriction site within both IS copies. In further experiments, the internal *Hin*dIII-

TABLE 2. Characterization of Fix$^-$ mutants selected after random Tn*5* mutagenesis

Strain	Site of Tn*5* insertion[a]	Size (kb) of *Eco*RI fragments carrying Tn*5*	Mol size increase of *Eco*RI fragments hybridizing with pEK5022 (kb)
AK1485	C	12	8.5 → 11
AK1486	P	8.5	8.5 → 11
AK1487	P	20	NC[b]
AK1488	P	15	4.8 → 7.5
AK1489	P	13	12 → 15
AK1490	P	15	NC
AK1491	P	15	8.5 → 15
AK1492	C	12	NC
AK1493	P	8	8.5 → 11
AK1494	C	12	8.5 → 11

[a] C, Chromosome; *P*, plasmid.
[b] NC, No change.

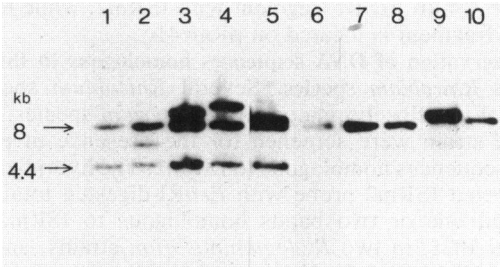

FIG. 2. Hybridization of ^{32}P-labeled ISRm2-specific *Hin*dIII-*Pst*I fragment to *Eco*RI-digested total DNAs from symbiotic mutants, wild-type *R. meliloti* 41, and several other *Rhizobium* species. Lanes: 1, wild-type *R. meliloti* 41; 2, AK1488; 3, AK1486; 4, AK1489; 5, ZB308; 6, ZB128; 7, *R. leguminosarum* 897; 8, *R. leguminosarum* 1015; 9, *R. trifolii* 24K/56; 10, *R. meliloti* 1021.

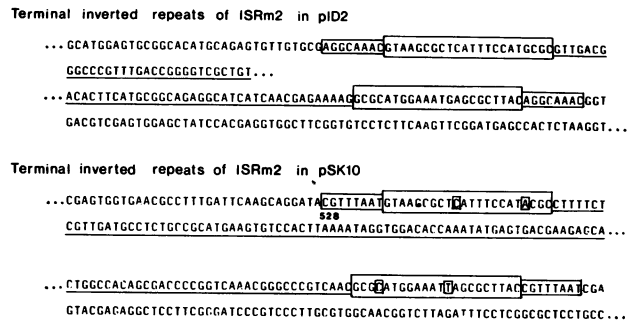

FIG. 3. DNA sequences of the terminal inverted repeats of ISRm2 from pID2 and pSK10. The target sequences and terminal repeats are shown in boxes. ISRm2 sequences are underlined. Short adjacent wild-type sequences at the 5′ and 3′ ends are also demonstrated. The number below the sequence from pSK10 indicates the position of the first base pair of the target sequence within the *hsnD* gene. The sequence data for *hsnD* are from Horvath et al. (18a).

*Pst*I fragment was used to prepare IS-specific hybridization probes.

Origin and localization of ISRm2 in *R. meliloti* 41. Two hybridizing *Eco*RI bands of 4.4 and 8 kb were detected when total DNA of the wild-type *R. meliloti* strain was hybridized with the internal *Hin*dIII-*Pst*I fragment of ISRm2, demonstrating the presence of two IS copies in the wild-type genome (Fig. 2). However, an additional hybridizing band of a different size was also visible when some of the Fix$^-$ and Nod$^-$ derivatives obtained after random Tn*5* mutagenesis were tested with the same probe, indicating the generation and insertion of a new copy of this IS element. Since this transposable element is of *R. meliloti* origin, we have designated it as ISRm2.

To localize the two copies of ISRm2 in the wild-type *R. meliloti* 41 genome, chromosomal DNA and plasmids were separated by gel electrophoresis and hybridized with the labeled internal fragment of ISRm2. One copy of the IS element is located on pRme41*a*, as demonstrated by the hybridization of this plasmid to the probe (data not shown). Since the other two plasmids pRme41*b* and pRme41*c* comigrate in 0.7% agarose gel, ZB138, a mutant derivative having a large deletion in pRme41*b*, was hybridized with the same probe. The second hybridizing band in ZB138 appeared at the level of pRme41*c*, demonstrating the presence of the second ISRm2 copy on that plasmid (data not shown).

The *Eco*RI-digested total DNA of the mutant ZB129, lacking pRme41*a*, did not reveal a hybridizing 4.4-kb fragment (Fig. 2). Therefore, we concluded that pRme41*a* carries the 4.4-kb *Eco*RI fragment with ISRm2, while the 8-kb *Eco*RI fragment is located on pRme41*c*.

Conservation of DNA sequences homologous to ISRm2 in various *Rhizobium* species. Several *Rhizobium* species, a strain of the closely related *Agrobacterium* species, and an *E. coli* strain were screened for the presence of possible DNA sequences homologous to ISRm2. The hybridization of the labeled ISRm2 probe with *Eco*RI-digested total DNAs revealed one or two bands homologous to ISRm2 in *R. meliloti* 1021, in two *R. leguminosarum* strains, and in *R. trifolii* 24K/56 (Fig. 2). However, hybridization of the probe to the other *Rhizobium* strains tested was less intense than to the parent strain, *R. meliloti* 41. In *R. leguminosarum* 248, *Agrobacterium tumefaciens* Ach5, and *E. coli* HB101, no sequences homologous to ISRm2 probe were detected (not shown).

Arrangement of ISRm2 copies in nitrogen-fixing bacteroids. The transposition of ISRm2 into DNA regions determining both nodulation and fixation functions was observed (e.g., for mutants AK1488 and ZB308). Since symbiotic functions were affected by ISRm2 insertions, the possible involvement of ISRm2 transposition in symbiotic nitrogen fixation processes was examined. *Eco*RI-digested total DNAs from the free-living bacteria and symbiotically effective root nodules were hybridized with a highly labeled (14) internal ISRm2 fragment. The hybridization pattern of nodule DNA was identical with that of the bacterial DNA (data not shown), suggesting that no ISRm2 transposition into new locations occurred.

Structure of ISRm2 and its integration sites. Perfect or nearly perfect terminal inverted repeats of about 10 to 40 bp are characteristic of the structure of IS elements (20). Insertion into new sites is accompanied by the duplication of a short sequence of target DNA, and, as a result, the IS sequence is flanked by direct repeats of the target DNA.

To look for such DNA sequences in ISRm2 and in the flanking regions, we cloned the termini of the IS element together with the adjacent wild-type sequences from both pID2 and pSK10 (Fig. 1). The exact sites of insertions were determined by comparing these sequences with those of the wild-type fragments.

In mutant ZB308, the ISRm2 was inserted into the nodulation gene *hsnD* coding for a 29-kilodalton (kDa) protein, which is involved in the host specificity of nodulation (Fig. 1). Since the nucleotide sequence of *hsnD* was determined previously in our laboratory (18a), we were able to locate ISRm2 within the *hsnD* protein-coding region at position +536. A 22-bp inverted repeat was found at the ends of ISRm2, and the insertion generated an 8-bp duplication of the target sequence. The left and right inverted repeats have two mismatches (Fig. 3).

The Fix$^-$ mutant AK1488 carries ISRm2 between the *Hin*dIII and *Bam*HI restriction sites shown in Fig. 1. The corresponding *Eco*RI fragment from *R. meliloti* 1021 contains the *fixABCX* genes and part of *nifA* (8, 12). Since in *R. meliloti* 41, the DNA sequence around the site of insertion was not available, we sequenced the *Hin*dIII-*Bam*HI fragment (clones pSK12 and pSK15) and a fragment upstream of the *Hin*dIII site (clone pID23; Fig. 1). Analysis of the DNA sequence data revealed a 306-bp-long open reading frame (ORF) in this region, and the ISRm2 insertion was located in this ORF at the position indicated in Fig. 4. At the termini of this ISRm2 copy, 22-bp-long perfect inverted repeats were found. Both the left and right repeated sequences have one mismatch compared with the corresponding terminal repeats of the other ISRm2 copy in pSK10 (Fig. 3).

A new symbiotic gene: *fixX*. As a result of the ISRm2 insertion, a Fix$^-$ phenotype was observed in the mutant AK1488, indicating that the short ORF codes for a functional symbiotic gene. The amino acid sequence deduced from the ORF DNA sequence is shown in Fig. 4. The 98 amino acids define a polypeptide of 10.95 kDa. Six cysteines, four of which are arranged in the C-terminal part, are present in the molecule. Since the number and position of the cysteine residues and the low molecular mass of the protein are characteristic of bacterial ferredoxins, the amino acid sequence determined by the *R. meliloti fix* gene (designated as *fixX*) was compared with ferredoxins of different bacterial origin (Fig. 4). The most-conserved region was found around the characteristic cysteine residues.

The presence of a sequence cysteine-two amino acids-cysteine-two amino acids-cysteine in one part of the mole-

cule and a sequence cysteine-proline in the other part is required for formation of an iron-sulfur binding cluster (7). On the basis of our results, we suggest that *fixX* codes for a ferredoxin, in which four cysteine residues (66, 69, 72, and 46) participate in the binding of one iron-sulfur cluster. The remaining two cysteines may form a disulfide bridge.

DISCUSSION

ISRm2, a 2.7-kb-long IS element, has been shown to be present in *R. meliloti* 41. The presence of another IS sequence, ISRm1 (1.4 kb), in strain *R. meliloti* 1021 was previously reported (33). Hybridization of an internal restriction fragment of ISRm2 to the total DNA of *R. meliloti* 1021 revealed one hybridizing band (Fig. 2). This homology, however, is not due to the presence of similar DNA sequences in ISRm1 and ISRm2, since *R. meliloti* 1021 carries 10 copies of ISRm1 (33). The differences in size and in the restriction map also indicate that ISRm2 is a new element that is not related to ISRm1.

The transposition properties of ISRm2 and ISRm1 are similar. In both strains, the generation of new IS copies and the insertion into new locations were observed when symbiotic mutants were screened after random Tn*5* mutagenesis. Whether the transposition of Tn*5* has any role in the insertion of *R. meliloti* IS elements into new sites remains to be studied. Since the inverted repeat sequences are different at the termini of ISRm2 and Tn*5* (9), it is unlikely that a Tn*5*-encoded enzyme (transposase) plays any role in the transposition of ISRm2.

In constrast to the situation with ISRm1, hybridization revealed DNA sequence homology to ISRm2 in several *Rhizobium* species (Fig. 2). Whether these homologous regions are carried by plasmids or by chromosomes is not yet known. No DNA homology was detected with *R. leguminosarum* 248, *A. tumefaciens* Ach5, or *E. coli* HB101.

ISRm2 carries 22-bp-long inverted repeats at its termini. In one copy (cloned into pID2), perfect inverted repeats were found, while in a second copy (in pSK10), two mismatches were detected (Fig. 3). Therefore, the left and right terminal repeats in pSK10 each differ from the terminal repeats in pID2 by 1 bp. Moreover, in the copy cloned into pSK10, one *Xho*I restriction site was missing. These variations in DNA sequence may reflect differences between the two IS copies present in the wild-type genome. Sequence comparisons revealed a homology of several base pairs between the inverted repeats of ISRm2 and the inverted repeats of previously studied transposable elements which generate 9-bp target sequence duplications (9). The longest conserved sequence in IS*1*, a representative of these elements, is -CTGATTT-, nearly identical to -CTCATTT- found in ISRm2.

We show that insertion of ISRm2 causes an 8-bp sequence duplication at target sites, which is not common among IS elements (20). Only IS*1* and a variant of it are known to generate either an 8- or 9-bp duplication (19). The target sequences of ISRm2 carry A+T-rich regions (-CGTTTAAT- and -AGGCAAAC-), also characteristic of the IS*1* (29) and IS*10* (22) target sequences.

As we concluded from DNA sequence data, the Nod$^-$ phenotype of mutant ZB308 was due to ISRm2 insertion into the protein-coding region of the *hsnD* gene. Transposon Tn*5* insertions into *hsnD* also resulted in the loss of nodulation ability on the host plant alfalfa (18a). In the Fix$^-$ mutant AK1488, the *Hin*dIII-*Bam*HI fragment, where ISRm2 was localized, is situated between the coding regions of *fixABC*

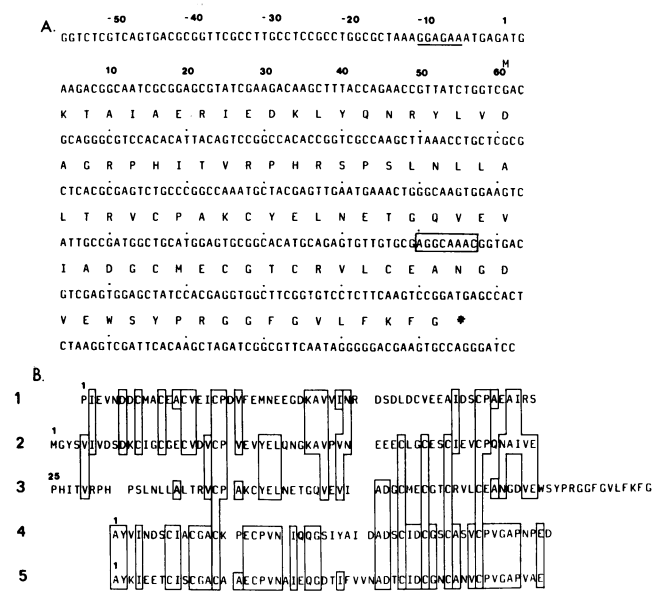

FIG. 4. (A) DNA sequence of *fixX* from *R. meliloti* 41 and the deduced amino acid sequence. The 8-bp target of the ISRm2 insertion is boxed. A putative ribosome-binding site upstream of the ATG start codon is underlined. (B) Comparison of the partial sequence of the *fixX* product from *R. meliloti* 41 with bacterial ferredoxins. Homologous sequences are boxed. Sources of ferredoxins are as follows: 1, *Desulfovibrio gigas* (7); 2, *D. desulfuricans* Norway (18); 3, *R. meliloti* 41; 4, *Peptococcus aerogenes* (38); 5, *Rhodospirillum rubrum* (37).

and *nifA* (8) (Fig. 1). Recently, a short ORF has been detected between *fixC* and *nifA* in *R. meliloti* 1021 (12; A. Puhler, personal communication). A 306-bp ORF was also found in the identical position in *R. meliloti* 41. According to DNA sequence data, one of the target sites of ISRm2 was located within this ORF. The insertion resulted in a Fix$^-$ phenotype, suggesting that this ORF corresponds to a functional gene (designated as *fixX*) that participates in symbiotic processes. On the basis of similarities to bacterial ferredoxins, we conclude that *fixX* codes for a ferredoxin. Bacterial ferredoxins contain either one or two iron-sulfur clusters bound through one or two sets of four cysteine residues (30). The arrangement of four cysteines in the *fixX* protein, cysteine-proline in the N-terminal part and three cysteines, each separated by two amino acids, in the C-terminal part of the protein, fulfils the basic requirements for binding one Fe-S cluster (7). In contrast, other ferredoxins, having a single Fe-S center, carry the three cysteines at the N-terminal part of the molecule (37). The amino acid sequences of the *fixX* products from *R. meliloti* 41 and 1021 are highly homologous (12; Puhler, personal communication). A difference was found at position 62, where a threonine in *R. meliloti* 1021 was replaced by an isoleucine in *R. meliloti* 41. Like other ferredoxins, the *fixX* gene product probably functions as an electron carrier. Therefore, it may be functionally related to the *fixABC* operon, which is assumed to code for electron transport proteins (12, 17).

In the wild type *R. meliloti* 41, the two ISRm2 copies carried by the 4.4- and 8-kb *Eco*RI fragments are located on plasmids pRme41*a* and pRme41*c*, respectively. New ISRm2 copies generated by transposition were localized on the third plasmid, pRme41*b*. In 6 of 10 mutants, the insertion occurred within a 49-kb region of this megaplasmid (shown by

hybridization with pEK5022) (Table 2). It is interesting that in four of the six mutants mentioned, ISRm2 transposed into an 8.5-kb *Eco*RI fragment adjacent to the fragment carrying the *fixABC* genes. It is difficult to conclude whether ISRm2 integration shows a preference for the *nod-nif* region of the megaplasmid, since after random Tn*5* mutagenesis, the Tn*5* insertion derivatives were screened for the Fix$^-$ or Nod$^-$ phenotype. Our results, however, are in agreement with the data concerning ISRm1, which was reported to transpose into the same *nod-nif* region of *R. meliloti* 1021. We have no data yet about the possible integration frequency of ISRm2 into nonsymbiotic regions of the *Rhizobium* genome.

Repeated sequences of *B. japonicum* RSRjα and RSRjβ have not been found to transpose, but, interestingly, some of them are clustered around the nitrogenase genes. The repeated sequences of *B. japonicum* had the same arrangement when the bacterial and nodule bacteroid DNAs were compared (21). Similarly, the identical hybridization pattern of ISRm2 in nodules and in a free-living *R. meliloti* strain suggests that the direct involvement of this element in symbiotic nitrogen fixation is unlikely.

ACKNOWLEDGMENTS

We are grateful to F. Ausubel and A. Puhler for providing sequence data prior to publication, to G. B. Kiss for providing a radioactive probe, to B. Horvath and C. Pazmany for their help in DNA sequencing, to C. Bachem for computer analysis, and to D. Gerhold for critical reading of the manuscript. We thank J. Borzi and Z. Liptay for technical assistance, K. Szabo for typing the manuscript, and B. Dusha for making the photos.

S. Kovalenko was the recipient of a fellowship from the International Training Course of the Hungarian Academy of Sciences.

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