

## SUPPLEMENTARY MATERIAL AND METHODS

### Gene deletions and mutant complementations

Gene deletions were performed using pJQ200mp19 derivatives containing ~350 to 450 bp regions flanking the gene(s) to be deleted: *ku1* (SMa0426), *ku2* (SMb20686), *ku3 ku4* (SMb21406-SMb21407), *ligD1* (SMa0414-SMa0417), *ligD2* (SMb20685), *ligD3* (SMb21044), *ligD4* (SMc03959). Open reading frames-flanking DNA fragments were amplified by PCR using *S. meliloti* GMI11495 genomic DNA as template and the oligonucleotides listed in Table S2 as primers, and individually cloned into pGEM-T. These regions were then subsequently juxtaposed as *SalI*-*Bam*HI and *Bam*HI-*SacI* fragments into *SalI*-*SacI*-digested pJQ200mp19. Plasmids were introduced in *S. meliloti* by electrotransformation as described (1). Single-crossover genomic integration of each pJQ200mp19 derivative was generated by selecting for Gm resistance. The resulting strains were then propagated in the absence of antibiotic, and cells having lost the plasmid by a second recombination event were selected by plating on LBMC supplemented with 5% sucrose (Suc). Suc<sup>R</sup> Gm<sup>S</sup> colonies were screened by PCR analysis using primers indicated in Table S2. Multiple mutants were constructed by the introduction of successive deletions as indicated in Table S3.

For complementations, *ligD2* and *ku2* genes, including their own promoters, were amplified by PCR using *S. meliloti* GMI11495 genomic DNA as template and OCB1444-OCB1445 and OCB1502-OCB1503 as primers, respectively, and cloned into pJET1.2. *ligD2* was extracted from pJET1.2 by *Bgl*III digestion and subcloned into the pJQ200mp19-derivative pLS272 (see construction details below) in *Bam*HI. *ku2* was extracted from pJET1.2 by *Xho*I-*Xba*I digestions and subcloned into pLS272 in *Nsi*I-*Avr*II. Before *Xba*I or *Avr*II digestions, *Xho*I and *Nsi*I DNA ends were blunted using the T4 DNA polymerase. *ku2* and *ligD2* genes were introduced into the *S. meliloti* chromosome (*rhaS* gene) by a double recombination event as described above for gene deletions.

For *ku3* and *ku3-ku4* operon complementation, genes including their own promoters were amplified by PCR using *S. meliloti* GMI11495 genomic DNA as template and OCB1426-OCB1427 and OCB1426-OCB1428 as primers, respectively. For *ku4* complementation, PCR amplification was carried out with OCB1426-OCB1428 using CBT1811 (*S. meliloti* GMI11495  $\Delta ku3$ ) genomic DNA as template to produce a fragment containing *ku4* preceded by the promoter of the *ku3-ku4* operon. These fragments were cloned into pGEM-T before being subcloned into the pJQ200mp19 derivative pLS282-3 (see construction details below)

as *XbaI-BamHI* (*ku3* and *ku3-ku4* complementation) or *XbaI-NdeI* (*ku4* complementation) fragments.

### ***I-SceI* expression and restriction site integration**

For *I-SceI* expression, a plasmid derived from pQF was used to clone the *I-SceI* coding sequence under the control of a cumate inducible promoter. pQF was first modified by inserting in between the *AseI-SpeI* restriction sites a dsDNA adapter obtained by annealing oligonucleotides OCB1481 and OCB1482, giving pLS256-1. Using OCB1483 and OCB1484, a second adapter was then cloned into *BamHI-MfeI*-cut pLS256-1 giving pLS257-1. Then, an *NdeI-PstI* fragment containing the *I-SceI* coding sequence was purified from pDAI-*SceI* and subcloned into *AseI-NsiI*-cut pLS257-1, giving pLS273-25. Among the first *S. meliloti* clones carrying the *I-SceI* expressing plasmid but still having an intact *I-SceI* restriction site on the chromosome, several were streaked out on TY medium containing tetracycline and supplemented or not by 100  $\mu$ M cumate. All tested clones but one (containing pLS273-25\*) were able to grow on cumate-containing medium suggesting that this latter was still able to cleave *I-SceI* restriction site on the chromosome when induced with cumate. The plasmid from this cumate sensitive clone was extracted and sequenced, revealing a stop codon (TAC>TAG), leading to the synthesis of a truncated protein lacking the last 31 amino acids, presumably less active than WT form of I-SceI meganuclease.

To introduce an *I-SceI* restriction site into the *S. meliloti* chromosome, the first half of the *rhaS* coding sequence was PCR amplified using OCB1531 and OCB1532, generating a fragment flanked by *SacI* and *BamHI* restriction sites. The second half of *rhaS* was PCR amplified using OCB1533 and OCB1534 giving a fragment flanked by *BamHI* and *SalI* restriction sites and bearing an *I-SceI* restriction site in the vicinity of *BamHI*. These two fragments were individually cloned into pGEM-T before being juxtaposed into *SacI-SalI*-cut pJQ200mp19 as *SacI-BamHI* and *BamHI-SalI* fragments, giving pLS272-1. Addition of a second *I-SceI* restriction site and a multiple cloning site between the two halves of *rhaS* was performed by inserting an adapter into *BamHI*-cut pLS272-1. This adapter was obtained by annealing OCB1581 and OCB1582 and the orientation of adapter insertion was checked by PCR followed by *I-SceI* restriction. pLS282-3 contains an inverted orientation of the adapter, leading to two successive *I-SceI* sites followed by the multiple cloning site (*XhoI*, *XbaI*, *SmaI*, *XmaI*, *SpeI*, *MfeI*, *NdeI*).

**Plasmid constructions for  $\beta$ -galactosidase assays.** Plasmids were constructed from the *ku* and *ligD* upstream regions cloned into pGEM-T for gene deletions, and containing the gene promoters. Gene promoters were extracted from pGEM-T derivatives with *Bam*HI/*Nsi*I digestions and subcloned into pCZ962 in *Xba*I/*Nsi*I sites. Before the *Nsi*I digestion, *Bam*HI and *Xba*I DNA ends were blunted using the T4 DNA polymerase.

### **Plasmid-based NHEJ assay**

To measure NHEJ efficiency, several pBBR1MCS-5 derivatives were constructed. The 5' region of *lacZ* was amplified by PCR using pCZ962 as template and OCB1317-OCB1318 as primers, and cloned into pGEM-T. The *Sac*I/*Age*I *lacZ* 5' region was subcloned into pBBR1MCS-5 giving pDP59. The *lacZ* 3' region was extracted from pCZ962 with *Aat*II and *Asc*I digestions and subcloned into pDP59 in *Aat*II/*Mlu*I sites giving pDP62. The *sacB* gene was amplified by PCR using pJQ200mp19 as template and OCB1319-OCB1320 as primers, cloned into pGEM-T and subcloned in *Bam*HI, *Sma*I or *Pst*I in pDP62 giving respectively pDP63, pDP64 and pDP65.

The Tet<sup>R</sup> pBBR1MCS-5 derivative used for normalization of transformation efficiencies was constructed as follows. The *tetA tetR* region was amplified by PCR using pCZ962 as template and OCB1414-OCB1415 as primers, cloned into pGEM-T, and subcloned into pBBR1MCS-5 as a *Nco*I/*Bgl*II fragment (*Nco*I/*Bgl*II digestion removes the Gm<sup>R</sup> gene from pBBR1MCS-5).

### **DNA integration assays**

The DNA fragment conferring spectinomycin resistance was amplified from pHP45- $\Omega$  using OCB1535 and OCB1543, each one carrying a *Bst*XI restriction site contiguous to an external *Bam*HI restriction site. For each primer, the *Bst*XI restriction site was designed to generate a 3' protruding end compatible with the 3' protruding ends generated by *I-Sce*I. The PCR fragment was cloned into pGEM-T, and then subcloned into *Bam*HI-cut pBBR1MCS-5 giving pLS278-9.

## SUPPLEMENTARY TABLES

**Table S1:** Strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
<b>Strains</b>		
<i>Sinorhizobium meliloti</i>		
GMI11495	wild-type strain (Sm <sup>R</sup> ), Rm2011 background	(2, 3)
CBT1809	GMI11495 $\Delta ku1$ (SMa0426)	This work
CBT1810	GMI11495 $\Delta ku2$ (SMb20686)	This work
CBT1823	GMI11495 $\Delta ku34$ (SMb21406-21407)	This work
CBT1892	GMI11495 $\Delta ku12$	This work
CBT1893	GMI11495 $\Delta ku234$	This work
CBT1960	GMI11495 $\Delta ku123$	This work
CBT1961	GMI11495 $\Delta ku124$	This work
CBT1899	GMI11495 $\Delta ku1234$	(4)
CBT1938	GMI11495 $\Delta recA$	(4)
CBT1943	GMI11495 $\Delta recA \Delta ku1234$	(4)
CBT2183	GMI11495 $\Delta ku2 rhaS::ku2$	This work
CBT2381	GMI11495 $\Delta ku123 rhaS::ku3$	This work
CBT2383	GMI11495 $\Delta ku124 rhaS::ku4$	This work
CBT2385	GMI11495 $\Delta ku1234 rhaS::ku34$	This work
CBT2382	GMI11495 $\Delta ku1234 rhaS::ku3$	This work
CBT2384	GMI11495 $\Delta ku1234 rhaS::ku4$	This work
CBT1813	GMI11495 $\Delta ligD1$ (SMa0414-0417)	This work
CBT2162	GMI11495 $\Delta ligD2$ (SMb20685)	This work
CBT1811	GMI11495 $\Delta ku3$ (SMb21406)	This work
CBT1815	GMI11495 $\Delta ligD3$ (SMb21044)	This work
CBT1816	GMI11495 $\Delta ligD4$ (SMc03959)	This work
CBT2158	GMI11495 $\Delta ligD134$	This work
CBT2180	GMI11495 $\Delta ligD234$	This work
CBT2164	GMI11495 $\Delta ligD1234$	This work
CBT2172	GMI11495 $\Delta ligD2 rhaS::ligD2$	This work
CBT2000	GMI11495 $\Delta ku12 \Delta ligD2$	This work
CBT1962	GMI11495 $\Delta ku12 \Delta ligD4$	This work
CBT2005	GMI11495 $\Delta ku12 \Delta ligD24$	This work
CBT2082	GMI11495 $\Delta ku12 \Delta ligD234$	This work
CBT2120	GMI11495 $\Delta ku12 \Delta ligD1234$	This work
CBT2173	GMI11495 $rhaS::I-SceI$	This work
CBT2175	GMI11495 $\Delta ku2 rhaS::I-SceI$	This work
CBT2177	GMI11495 $\Delta ku1234 rhaS::I-SceI$	This work
CBT2496	GMI11495 $\Delta ligD1234 rhaS::I-SceI$	This work
CBT907	GMI11495 $rpoE2::hph$	(3)
CBT2003	GMI11495 $\Delta ku12 rpoE2::hph$	This work
<i>Escherichia coli</i>		
DH5 $\alpha$	F <sup>+</sup> $\Phi 80lacZ\Delta M15 \Delta(lacZYA-argF)$ U169 $recA1 endA1$ $hsdR17(r_k^-, m_k^+)$ $phoA supE44 thi-1 gyrA96 relA1 \lambda^-$	Invitrogen

## Plasmids

pGEM-T	Cloning vector (Amp <sup>R</sup> )	Promega
pJET1.2	Cloning vector (Amp <sup>R</sup> )	ThermoScientific
pJQ200mp19	Gene replacement vector (Gm <sup>R</sup> )	(5)
pBBR1MCS-5	Expression vector (Gm <sup>R</sup> )	(6)
pHP45-Ω	vector (Spec <sup>R</sup> )	(7)
pCZ962	Cloning vector (Tet <sup>R</sup> Amp <sup>R</sup> )	(8)
pDAI- <i>SceI</i>	<i>I-SceI</i> constitutive expression vector (Tet <sup>R</sup> )	(9)
pQF	Cumate inducible expression vector (Tet <sup>R</sup> )	(10)
pDP39	pJQ200mp19 derivative for <i>ku1</i> deletion	this work
pDP40	pJQ200mp19 derivative for <i>ku2</i> deletion	this work
pDP41	pJQ200mp19 derivative for <i>ku3</i> deletion	this work
pDP42	pJQ200mp19 derivative for <i>ku4</i> deletion	this work
pDP56	pJQ200mp19 derivative for <i>ku34</i> deletion	this work
pDP43	pJQ200mp19 derivative for <i>ligD1</i> deletion	this work
pDP113	pJQ200mp19 derivative for <i>ligD2</i> deletion	this work
pDP45	pJQ200mp19 derivative for <i>ligD3</i> deletion	this work
pDP46	pJQ200mp19 derivative for <i>ligD4</i> deletion	this work
pDP55	pJQ200mp19 derivative for <i>ligD2ku2</i> deletion	this work
pDP52	pJQ200mp19 derivative for <i>recA</i> deletion	this work
pLS272-1	pJQ200mp19 derivative for <i>I-SceI</i> insertion at the <i>rhaS</i> locus	this work
pLS282-3	pJQ200mp19 derivative for 2 <i>I-SceI</i> insertion at the <i>rhaS</i> locus	this work
pDP115	pLS272-1 derivative for <i>ligD2</i> insertion at the <i>rhaS</i> locus	this work
pDP116	pLS272-1 derivative for <i>ku2</i> insertion at the <i>rhaS</i> locus	this work
pLS289-9	pLS282-3 derivative for <i>ku3</i> insertion at the <i>rhaS</i> locus	this work
pLS290-13	pLS282-3 derivative for <i>ku4</i> insertion at the <i>rhaS</i> locus	this work
pLS294-8	pLS282-3 derivative for <i>ku34</i> insertion at the <i>rhaS</i> locus	this work
pDP66	pCZ962 derivative for measure of <i>ku34</i> promoter activity	this work
pDP67	pCZ962 derivative for measure of <i>ligD4</i> promoter activity	this work
pDP68	pCZ962 derivative for measure of <i>ku1</i> promoter activity	this work
pDP69	pCZ962 derivative for measure of <i>ku2</i> promoter activity	this work
pDP70	pCZ962 derivative for measure of <i>ligD2</i> promoter activity	this work
pDP92	pCZ962 derivative for measure of <i>ligD1</i> promoter activity	this work
pDP93	pCZ962 derivative for measure of <i>ligD3</i> promoter activity	this work
pDP58	pGEM-T derivative with <i>sacB</i>	this work
pDP62	pBBR1MCS-5 with complete <i>lacZ</i> gene	this work
pDP63	pDP62 with <i>sacB</i> inside <i>lacZ</i> in <i>BamHI</i> site	this work
pDP64	pDP62 with <i>sacB</i> inside <i>lacZ</i> in <i>SmaI</i> site	this work
pDP65	pDP62 with <i>sacB</i> inside <i>lacZ</i> in <i>PstI</i> site	this work
pDP90	pBBR1MCS-5 derivative (Tet <sup>R</sup> )	this work
pLS257-1	pQF derivative without 3' and 5' Flag	this work
pLS273-25	pLS257-1 derivative for <i>I-SceI</i> constitutive expression	this work
pLS273-25*	pLS273-25 derivative for <i>I-SceI</i> cumate inducible expression	this work
pLS278-9	pBBR1MCS-5 derivative with Spec <sup>R</sup> cassette	this work

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**Table S2:** Oligonucleotides used in this study.

Name	Sequence (5'→3') <sup>a</sup>	Target
Amplification of upstream and downstream regions of genes for deletions		
OCB1284	<b>GGATCCGGCCATGGCGACCTCCG</b>	fw <i>ku1</i> upstream
OCB1285	<b>GAGCTCCGACGGCCTTCTGCTTGG</b>	rev <i>ku1</i> upstream
OCB1286	<b>GTCGACGAAATCGGACCGTCCTTCC</b>	fw <i>ku1</i> downstream
OCB1287	<b>GGATCCGCCTTGC GGAAATCAGTCG</b>	rev <i>ku1</i> downstream
OCB1272	<b>GTCGACTAAAGACATCTGGCGGCAGG</b>	fw <i>ku2</i> upstream
OCB1273	<b>GGATCCTGCCATGGTTTTCTCCCTACG</b>	rev <i>ku2</i> upstream
OCB1274	<b>GGATCCGCCAAGAAGAAAGCCTGATGG</b>	fw <i>ku2</i> downstream
OCB1275	<b>GAGCTCGAGGCGACGTTTCCGGAGC</b>	rev <i>ku2</i> downstream
OCB1260	<b>GTCGACACGCGGTCAAGGCCACC</b>	fw <i>ku3</i> upstream
OCB1261	<b>GGATCCTGCCATGGCTAACTCCCTG</b>	rev <i>ku3</i> upstream
OCB1262	<b>GGATCCGCCAGCTAGGAAGGTGAACG</b>	fw <i>ku3</i> downstream
OCB1263	<b>GAGCTCCGCACGGTTCTAGCATCACG</b>	rev <i>ku3</i> downstream
OCB1264	<b>GTCGACAAGTCGCCGCAATCGCCC</b>	fw <i>ku4</i> upstream
OCB1265	<b>GGATCCC GCCATCGTTCACCTTCC</b>	rev <i>ku4</i> upstream
OCB1266	<b>GGATCCAAGTCTTAAGGAGGGATGGC</b>	fw <i>ku4</i> downstream
OCB1267	<b>GAGCTCCGTTGTGGTCCAGTACTGC</b>	rev <i>ku4</i> downstream
OCB1292	<b>GAGCTCCTAGAACGAGGAACCCGG</b>	fw <i>ligD1</i> upstream
OCB1293	<b>GGATCCCTGAGCCTCGCCGCTTGG</b>	rev <i>ligD1</i> upstream
OCB1294	<b>GGATCCGCTGCGTGAGCACCAGG</b>	fw <i>ligD1</i> downstream
OCB1295	<b>GTCGACGGCTCACTGCGCTCGGC</b>	rev <i>ligD1</i> downstream
OCB1268	<b>GAGCTCCTCGCCCATGCCGGAAACG</b>	fw <i>ligD2</i> upstream
OCB1269	<b>GGATCCC GTCGCCATGCGCCCAAGC</b>	rev <i>ligD2</i> upstream
OCB1525	<b>GGATCCC GCAAGCTGCTCGGCAAGC</b>	fw <i>ligD2</i> downstream
OCB1526	<b>GTCGACGTTTCCGCCTGCATCCCG</b>	rev <i>ligD2</i> downstream
OCB1280	<b>GGATCCGTCCACCCATCCAACCC</b>	fw <i>ligD3</i> upstream
OCB1281	<b>GAGCTCCAAAGACGGCAAGCTCACCC</b>	rev <i>ligD3</i> upstream
OCB1282	<b>GTCGACGTGCGGAAACGCATGGAGG</b>	fw <i>ligD3</i> downstream
OCB1283	<b>GGATCCAGCTAATCATCTGCCTGGG</b>	rev <i>ligD3</i> downstream
OCB1276	<b>CTCTTGTCGACGACGATAGC</b>	fw <i>ligD4</i> upstream
OCB1277	<b>GGATCCGGCCATGGATCGTCAGCC</b>	rev <i>ligD4</i> upstream
OCB1278	<b>GGATCCGTGCGAATGAGCCGGCCG</b>	fw <i>ligD4</i> downstream
OCB1279	<b>GAGCTCATCCTCCCGGAGAATGCC</b>	rev <i>ligD4</i> downstream
OCB1302	<b>GAGCTCCCGAGCATCTCGAAGAAGG</b>	fw <i>recA</i> upstream
OCB1303	<b>GGATCCGATATGTAATCGGGAACAACC</b>	rev <i>recA</i> upstream
OCB1304	<b>GGATCCC GATTTGTCTCTACGAGC</b>	fw <i>recA</i> downstream
OCB1305	<b>GTCGACGCTTCTCGAACCAAGCGC</b>	rev <i>recA</i> downstream
Screening of genomic deletions or insertions		
OCB1321	<b>GATGCTTGGCGATGATCCC</b>	fw screening of $\Delta ku1$
OCB1322	<b>GCCACTGGCATGACGTCG</b>	rev screening of $\Delta ku1$
OCB1323	<b>CGCAAGCTGCTCGGCAAGC</b>	fw screening of $\Delta ku2$
OCB1324	<b>GAGCGAAGAGATCTTCGAGC</b>	rev screening of $\Delta ku2$

OCB1325	TTCTGACGAGCGTGATCGC	fw screening of $\Delta ku34$
OCB1328	CTGCGGTCTGCTCCATGC	rev screening of $\Delta ku34$
OCB1329	ACTGCGGAATATCAGTCACC	fw screening of $\Delta ligD1$
OCB1330	GCCTCGTCTTGTTGCGTCC	rev screening of $\Delta ligD1$
OCB1331	GTGCGCCGACGCGATTCC	fw screening of $\Delta ligD2$
OCB1332	GCACCTCGTTGGGATTGCG	rev screening of $\Delta ligD2$
OCB1333	CTGGAATGCGCTCTTCGC	fw screening of $\Delta ligD3$
OCB1334	CGGATGAAGTTGGCGATGC	rev screening of $\Delta ligD3$
OCB1335	CCATTCCGCTCGTCTGCC	fw screening of $\Delta ligD4$
OCB1336	GCTCGGGAAGAGGACAGG	rev screening of $\Delta ligD4$
OCB1339	TGACGAGCAGACGCTTGGC	fw screening of $\Delta recA$
OCB1340	CACACCGGCAGCTTCCGC	rev screening of $\Delta recA$
OCB1539	TCGGGCAGGAAGACGCAGC	fw screening of insertions in <i>rhaS</i>
OCB1547	CAAACACCCCAGCACGATCAG	rev screening of insertions in <i>rhaS</i>
OCB1579	ATAGACGGTGGCGACCAC	fw sequencing of insertions in <i>I-SceI</i>
OCB1580	GGCAACATGATCATCAAGC	rev sequencing of insertions in <i>I-SceI</i>

Amplification of genes for complementations

OCB1502	<b>AAGCTT</b> ACTCGCCTGATATTGCAGCC	fw <i>ku2</i> for <i>ku2</i> complementation
OCB1503	<b>TCTAGA</b> ATGTCCGACCGGCAAGCG	rev <i>ku2</i> for <i>ku2</i> complementation
OCB1444	<b>TCTAGAC</b> GCAAGGCTTATGAGAAGG	fw <i>ligD2</i> for <i>ligD2</i> complementation
OCB1445	<b>GGATCC</b> GGCACTACGATCACATCTGC	rev <i>ligD2</i> for <i>ligD2</i> complementation
OCB1426	<b>TCTAGA</b> ACGCGGTCAAGGCCACC	fw <i>ku3</i> for <i>ku3/ku4</i> complementations
OCB1427	<b>GGATCC</b> CGCCATCGTTCACCTTCC	rev <i>ku3</i> for <i>ku3</i> complementation
OCB1428	<b>GGATCC</b> GCTTCCGCTATCGACCACC	rev <i>ku4</i> for <i>ku4</i> complementation

Constructions for transformation assays

OCB1414	<b>CCATGGT</b> GAGATAGATCCGTCAATTCC	fw <i>tetA tetR</i> (pCZ962)
OCB1415	<b>AGATCTG</b> CTCTGCTGTAGTGAGTGG	rev <i>tetA tetR</i> (pCZ962)
OCB1319	<b>CTGCAGGATCCC</b> GGGGATCGACTCTAGC TAGAGG	fw <i>sacB</i> (pJQ200mp19)
OCB1320	<b>CTGCAGGATCCC</b> GGGGATATCGACCCA AGTACCG	rev <i>sacB</i> (pJQ200mp19)
OCB1317	<b>GAGCTCC</b> GTCGTTTTTACAACGTCGTG	fw <i>lacZ</i> N-ter (pCZ962)
OCB1318	<b>ACCGGTAC</b> GCGTGAGTGGCAACATGGAA ATCG	rev <i>lacZ</i> N-ter (pCZ962)
OCB1531	<b>GAGCTCA</b> AGTCCTGAAATCATTGATGG	fw 5' <i>rhaS</i> ( <i>I-SceI</i> addition)
OCB1532	<b>GGATCCCC</b> ACGTATTCTGATTGGTCC	rev 5' <i>rhaS</i> ( <i>I-SceI</i> addition)
OCB1533	<b>GGATCCTAGGGATAACAGGGTAAT</b> GGA TCGCCGAAATGAAGAAGG	fw 3' <i>rhaS</i> ( <i>I-SceI</i> addition)
OCB1534	<b>GTCGAC</b> AGAAGATCTTCGCGAATTCC	rev 3' <i>rhaS</i> ( <i>I-SceI</i> addition)
OCB1535	<b>GGATCCCCAAATAATTGG</b> AGCTTAGTAA AGCCCTCGC	fw <i>spec<sup>R</sup> BstXI</i> DNA end (pHP45- $\Omega$ )
OCB1543	<b>GGATCCCCAATTATTTGG</b> GCCTGTTCCG TTCGTAAGC	rev <i>spec<sup>R</sup> BstXI</i> DNA end (pHP45- $\Omega$ )
OCB1581	GATCCATATGCAATTGACTAGTCCCGGGT CTAGACTCGAGTAGGGATAACAGGGTAA T	fw linker mcs + <i>I-SceI</i> addition (pLS272-1 <i>Bam</i> HI site)

OCB1582	GATCATTACCCTGTTATCCCTACTCGAGT CTAGACCCGGGACTAGTCAATTGCATATG	rev linker mcs + <i>I-SceI</i> addition (pLS272-1 <i>Bam</i> HI site)
Screening of NHEJ repair infidelity		
M13 rev	GGAAACAGCTATGACCAT	Universal primer
OCB1043	AAGGGGGATGTGCTGCAAGG	pBBR1MCS-5 and derivatives (upstream multiple cloning site)
OCB1178	CGTGCCTTCATCCGTTTCC	pBBR1MCS-5 and derivatives (downstream multiple cloning site)

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<sup>a</sup>restriction sites are indicated in bold letters. *I-SceI* and *Bst*XI sites are underlined.



**Table S3:** Multiple mutant constructions.

Strains	Description
CBT1809	<i>ku1</i> deletion in GMI11495 (WT)
CBT1810	<i>ku2</i> deletion in GMI11495 (WT)
CBT1813	<i>ligD1</i> deletion in GMI11495 (WT)
CBT1815	<i>ligD3</i> deletion in GMI11495 (WT)
CBT1816	<i>ligD4</i> deletion in GMI11495 (WT)
CBT1822	<i>ligD2</i> and <i>ku2</i> deletion in GMI11495 (WT)
CBT1823	<i>ku34</i> deletion in GMI11495 (WT)
CBT1892	<i>ku1</i> deletion in CBT1810
CBT1893	<i>ku34</i> deletion in CBT1810
CBT1899	<i>ku1</i> deletion in CBT1893
CBT1938	<i>recA</i> deletion in GMI11495
CBT1943	<i>recA</i> deletion in CBT1899
CBT1946	<i>ku4</i> deletion in CBT1810
CBT1957	<i>ku3</i> deletion in CBT1810
CBT1960	<i>ku1</i> deletion in CBT1957
CBT1961	<i>ku1</i> deletion in CBT1946
CBT1962	<i>ligD4</i> deletion in CBT1892
CBT1997	<i>ku2</i> deletion in CBT907 ( <i>rpoE2::hph</i> )
CBT2000	<i>ku1</i> deletion in CBT1822
CBT2003	<i>ku1</i> deletion in CBT1997
CBT2005	<i>ligD4</i> deletion in CBT2000
CBT2082	<i>ligD3</i> deletion in CBT2005
CBT2119	<i>ligD1</i> deletion in CBT1815
CBT2120	<i>ligD1</i> deletion in CBT2082
CBT2158	<i>ligD4</i> deletion on CBT2119
CBT2162	<i>ligD2</i> deletion in GMI11495 (WT)
CBT2163	<i>ligD2</i> deletion in CBT1816
CBT2164	<i>ligD2</i> deletion in CBT2158
CBT2180	<i>ligD3</i> deletion in CBT2163

**Table S4:** One way Anova detailed analysis values.

Figure	DNA end	total n values	F value	DFn	DFd	P value
Fig.1A	5' end ( <i>Bam</i> HI)	38	40.52	7	30	<0.0001
Fig.1A	Blunt end ( <i>Sma</i> I)*	27	3.831	4	22	0.0165
Fig.1A	3' end ( <i>Pst</i> I)	43	8.068	7	35	<0.0001
Fig.1B	5' end ( <i>Bam</i> HI)	24	64.42	4	19	<0.0001
Fig.1B	Blunt end ( <i>Sma</i> I)*	19	1.437	2	16	0.2668
Fig.1B	3' end ( <i>Pst</i> I)	26	5.24	4	21	0.0044
Fig.2A	5' end ( <i>Bam</i> HI)	40	71.58	6	33	<0.0001
Fig.2A	Blunt end ( <i>Sma</i> I)*	32	39.16	4	27	<0.0001
Fig.2A	3' end ( <i>Pst</i> I)	45	15.59	6	38	<0.0001
Fig.2B	5' end ( <i>Bam</i> HI)	67	110.2	13	53	<0.0001
Fig.2B	Blunt end ( <i>Sma</i> I)*	45	29.07	8	36	<0.0001
Fig.2B	3' end ( <i>Pst</i> I)	70	35.21	13	56	<0.0001
Fig.3A	5' end ( <i>Bam</i> HI)	25	191.7	6	18	<0.0001
Fig.3A	Blunt end ( <i>Sma</i> I)*	16	11.29	4	11	0.0007
Fig.3A	3' end ( <i>Pst</i> I)	25	50.2	6	18	<0.0001
Fig.3B	5' end ( <i>Bam</i> HI)	25	37.55	5	19	<0.0001
Fig.3B	Blunt end ( <i>Sma</i> I)*	18	39.55	3	14	<0.0001
Fig.3B	3' end ( <i>Pst</i> I)	24	10.82	5	18	<0.0001
Fig.4A	-	48	504.8	15	32	<0.0001
Fig.4B	-	36	450.8	11	24	<0.0001
Fig.4C	5' end ( <i>Bam</i> HI)	27	33.91	3	23	<0.0001
Fig.4C	Blunt end ( <i>Sma</i> I)*	n.a	n.a	n.a	n.a	n.a
Fig.5A	-	59	17.98	6	52	<0.0001
Fig.5B	-	24	48.98	7	16	<0.0001

\* for blunt end repair, strains unable to give colony were excluded from the test (treshold values reported on related figures instead of plot data).

**Table S5:** Fidelity of repair events.

Condition & genotype	Fidelity (%) <sup>a</sup>			Total number of colonies		
	<i>Bam</i> HI	<i>Sma</i> I	<i>Pst</i> I	<i>Bam</i> HI	<i>Sma</i> I	<i>Pst</i> I
<b><i>Log phase 28°C</i></b>						
WT	94.0	78.5	98.5	6578	177	6894
$\Delta$ <i>recA</i>	91.5	83.3	98.6	199	6	138
$\Delta$ <i>ku1</i>	86.4	83.3	98.8	1481	24	1212
$\Delta$ <i>ku2</i>	100.0	NA	100.0	26	0	415
$\Delta$ <i>ku34</i>	88.7	72.4	97.2	2308	29	1153
$\Delta$ <i>ku1234</i>	100.0	NA	100.0	20	0	380
$\Delta$ <i>ku1234</i> $\Delta$ <i>recA</i>	100.0	NA	100.0	93	0	170
$\Delta$ <i>ligD2</i>	100.0	NA	100.0	22	0	640
$\Delta$ <i>ligD134</i>	92.0	88.2	98.4	1391	17	610
$\Delta$ <i>ligD1234</i>	100.0	NA	100.0	20	0	524
<b><i>Stationary phase</i></b>						
WT	89.2	73.0	98.5	21341	1562	20063
$\Delta$ <i>ku1</i>	82.1	71.1	99.2	8244	646	11728
$\Delta$ <i>ku2</i>	95.1	67.8	97.0	1051	270	908
$\Delta$ <i>ku34</i>	83.2	58.8	89.3	7171	719	2430
$\Delta$ <i>ku234</i>	100.0	NA	98.8	55	0	2550
$\Delta$ <i>ku1234</i>	100.0	NA	100.0	58	0	727
<b><i>Log phase 40°C</i></b>						
WT	92.4	79.5	99.0	36035	2475	55006
$\Delta$ <i>ku1</i>	71.8	72.4	94.9	2050	4176	3702
$\Delta$ <i>ku2</i>	66.2	67.7	90.4	130	155	539
$\Delta$ <i>ku12</i>	87.1	69.3	83.7	3035	940	2881
$\Delta$ <i>ku34</i>	95.5	79.2	98.1	32078	1606	40628
$\Delta$ <i>ku1234</i>	100.0	NA	99.5	8	0	367
$\Delta$ <i>ligD1234</i>	100.0	NA	100.0	124	0	2050
$\Delta$ <i>ku12</i> $\Delta$ <i>ligD2</i>	74.5	63.4	80.4	987	1033	1400
$\Delta$ <i>ku12</i> $\Delta$ <i>ligD4</i>	98.2	36.8	98.4	739	19	624
$\Delta$ <i>ku12</i> $\Delta$ <i>ligD24</i>	100.0	0.0	99.9	19	56	950
$\Delta$ <i>ku12</i> $\Delta$ <i>ligD234</i>	100.0	NA	100.0	10	0	165
$\Delta$ <i>ku12</i> $\Delta$ <i>ligD1234</i>	100.0	NA	100.0	14	0	396

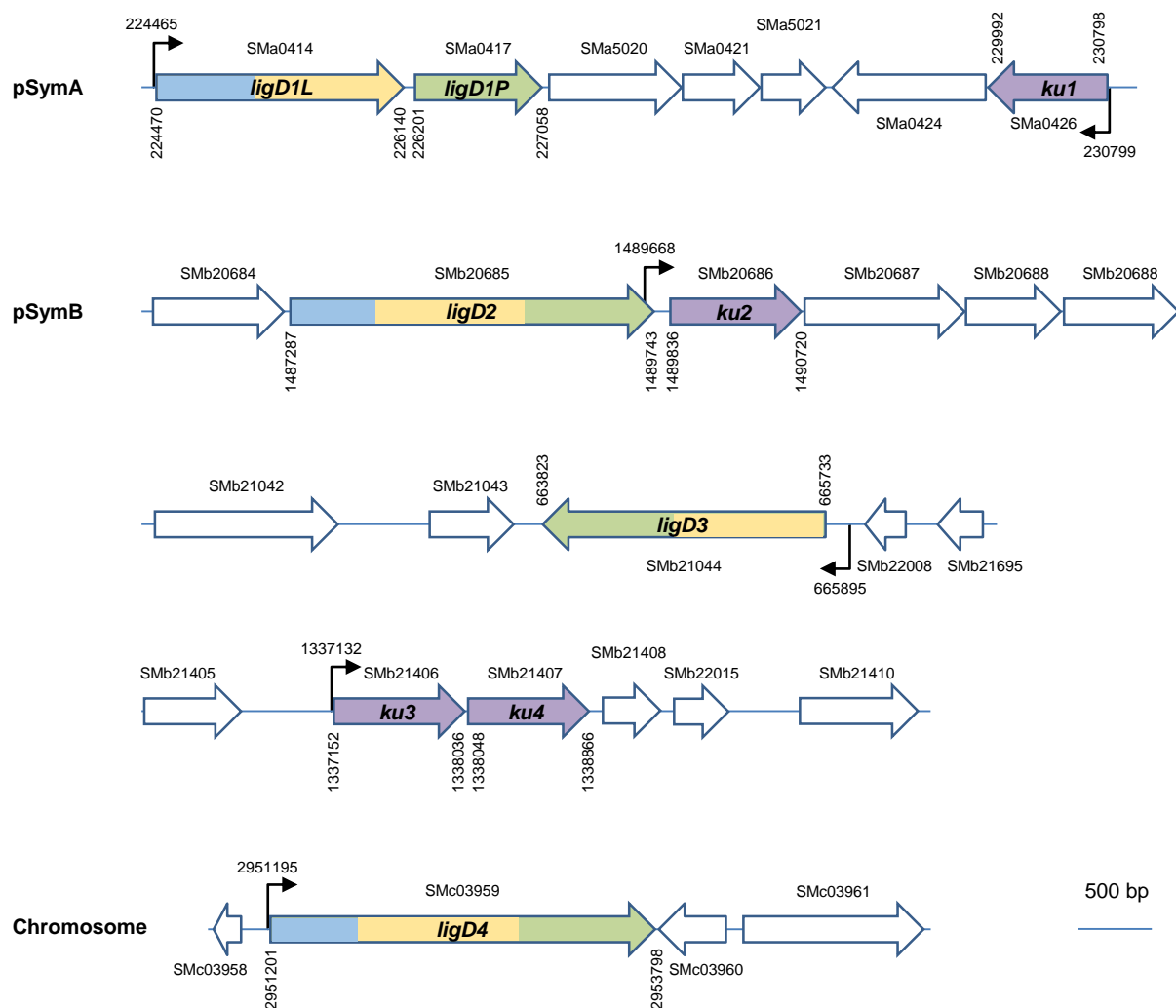
<sup>a</sup>Fidelity was calculated as the ratio of blue (Lac<sup>+</sup>) vs total colony numbers

**Table S6:** Fidelity of repair events: two-sided Fisher tests

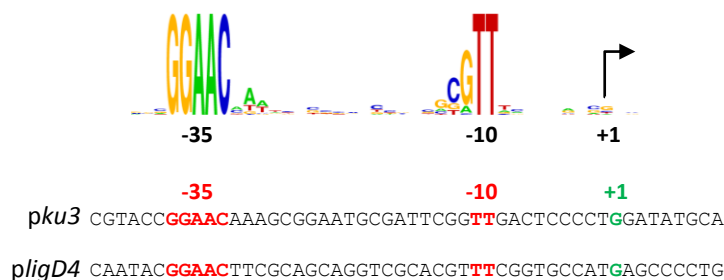
Condition & Genotype	<i>Bam</i> HI		P value	<i>Pst</i> I		P value
	Blue (Lac <sup>+</sup> )	White (Lac <sup>-</sup> )		Blue (Lac <sup>+</sup> )	White (Lac <sup>-</sup> )	
<i>Log phase 28°C</i>						
WT	6185	393	0.0098	6788	106	<0.0001
<i>Δku2+ΔligD2+Δku1234+ΔligD1234</i>	88	0		1959	0	
<i>Stationary phase</i>						
WT	19026	2315	0.0023	19769	294	<0.0001
<i>Δku1234</i>	58	0		727	0	
<i>Δku2</i>				881	27	0.0014
<i>Δku234</i>				2518	32	
<i>Δku2</i>				881	27	<0.0001
<i>Δku1234</i>				727	0	
<i>Log phase 40°C</i>						
WT	33305	2730	<0.0001	54440	566	<0.0001
<i>Δku1234+ΔligD1234</i>	132	0		2414	3	
<i>Δku2</i>				487	52	<0.0001
<i>Δku1234</i>				365	3	
<i>Δku12</i>				2411	470	<0.0001
<i>Δku1234</i>				365	2	
<i>Δku12 ΔligD2</i>				1125	275	<0.0001
<i>Δku12 ΔligD24+Δku12 ΔligD234+</i>				1510	1	
<i>Δku12 ΔligD1234</i>						
<i>Δku12 ΔligD4</i>				614	10	<0.0001
<i>Δku12 ΔligD24+Δku12 ΔligD234+</i>				1510	1	
<i>Δku12 ΔligD1234</i>						

## SUPPLEMENTARY FIGURES

**A**



**B**



**Figure S1.** Genomic context of *ku* and *ligD* genes in *Sinorhizobium meliloti* (**A**) Organization of the *ku* and *ligD* genes on the *S. meliloti* replicons (the chromosome and the two megaplasmids pSymA and pSymB). The arrows represent ORFs and their directions of transcription. Coordinates of each *ligD* and *ku* ORF are noted according to *S. meliloti* GMI11495 strain annotation (<https://iant.toulouse.inra.fr/S.meliloti2011>). When available, transcription start site is indicated for *ku* and *ligD* genes (black arrow with coordinate). Predicted nuclease (blue), ligase (yellow) and polymerase (green) domains of LigD proteins are indicated on the corresponding genes. Adapted from (11), with modifications. (**B**) Putative RpoE2 binding motifs in *ku3* and *ligD4* promoters. Top: consensus sequence of promoter motifs recognized by RpoE2 (adapted from previous work, 12); bottom: promoter sequences of *ku3* and *ligD4* genes.

<i>Bam</i> HI	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT CGCCGGCGAGATCTTGATCACCTAGGGGGCCGACGTCTTAAGCTATA	number
WT Log phase (28°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	26
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	large deletion	3
	total	30
WT Stationary phase (28°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	28
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	3
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	large deletion	1
	total	33
WT Log phase (40°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	4
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	large deletion	1
	total	7
<i>ku12</i> Log phase (40°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	8
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	AGTCCACC Δ27 GATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGAT Δ35 CGATACCGTCGACCT	1
	large deletion	3
	total	16
<i>Sma</i> I	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT CGCCGGCGAGATCTTGATCACCTAGGGGGCCGACGTCTTAAGCTATA	number
WT Log phase (28°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	8
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	3
	large deletion	1
	total	12
WT Stationary phase (28°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	20
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	7
	large deletion	2
	total	29
WT Log phase (40°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	2
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCC Δ32 TACCGT	1
	GATTAAG Δ92 GGGCTGCAGGAATTCGATAT	1
	large deletion	3
	total	7
<i>ku12</i> Log phase (40°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	4
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	CCCAG Δ113 GCCCGGT	1
	CCCAGTACGA Δ91 TACCGTCG	1
	large deletion	2
	total	10

<i>Pst</i> I	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT CGCCGGCGAGATCTTGATCACCTAGGGGGCCGACGTCTTAAGCTATA	number
WT Log phase (28°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	3
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	large deletion	3
	total	7
WT Stationary phase (28°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	2
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	3
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GGGGTTTTΔ78 GAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	large deletion	8
	total	17
WT Log phase (40°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	2
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTG Δ34 CCTCGAAAAA	1
	large deletion	2
	total	7
<i>ku12</i> Log phase (40°C)	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	5
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	2
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATATCAA	1
	GCGGCCGCTCTAGAACTAGTGGATCC <sup>▼</sup> CCCCGGGCTGCAGGAATTCGATAT	1
	total	10

**Figure S2.** Infidelity of NHEJ during linear plasmid repair in *Sinorhizobium meliloti*. Linear plasmid DNA generated by restriction digest with either *Bam*HI (5' overhang), *Sma*I (blunt) or *Pst*I (3' overhang) was used to transform WT (CBT707) and  $\Delta ku12$  (CBT1892) competent cells prepared from exponential phase cultures at 28°C (blue) or after heat stress at 40°C (red) or from stationary phase cultures (green). This figure shows the DNA sequence of junctions in Lac<sup>-</sup> colonies resulting from unfaithful repair events. Parental sequences are indicated as double-stranded DNA, whereas only the upper strand is shown for the sequenced DNA junctions. The restriction sites are in dark blue (with black triangles indicating the cut sites), the nucleotide additions in red, and the deletions in bold green. Large deletions are noted as numbers of deleted nucleotides (when known), or as large deletions when estimated to be > 100 nt by agarose gel electrophoresis of the plasmids. The number of events found is indicated in the right column. Note that insertions or deletions of 3 nucleotides were never found, presumably because they led to Lac<sup>+</sup> colonies. Sequencing of plasmid DNA from 51 randomly chosen blue (Lac<sup>+</sup>) colonies obtained upon transformation of various strains with linear plasmids carrying all types of DNA ends revealed the WT sequence in every case, showing that the level of false positives resulting from addition or deletion of nucleotide triplet(s) is less than 2%.

<i>I-SceI</i>		number
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA TCTTATGCACCCCTAGG <b>ATCCCTATTGTCC</b> ▲ <b>CATTACCT</b> AGCGGCTTTACT	
WT Log phase (28°C)	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	1
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	3
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	1
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	2
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>Δ251</b> ATGGCCGGACACGTGAAG	1
	GCGCTCGTCCCGGCACTGAA <b>Δ214</b> <b>CAGGGTAAT</b> GGATCGCCGAAATGA	1
	total	9
WT Log phase (40°C)	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	1
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	3
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	3
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	1
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>Δ343</b> CCTCATCAACGGTGCCGAA	1
	total	9
$\Delta ku2$ Log phase (40°C)	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	2
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	4
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	11
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	2
	AGAATACGTGGGGATCC <b>TAGGGATA</b> ▲ <b>CAGGGTAAT</b> GGATCGCCGAAATGA	3
	total	22

**Figure S3.** Infidelity of NHEJ during repair of chromosomal double-strand breaks. Competent cells of the *S. meliloti* strains CBT2173 (WT) and CBT2175 ( $\Delta ku2$ ) carrying a *I-SceI* site in the chromosome were prepared either at 28°C (blue) or 40°C (red) and were transformed with the *I-SceI*-expressing plasmid pLS273-25. This figure shows the DNA sequence of junctions in transformants carrying a *I-SceI* site resistant to restriction digest, which all result from unfaithful repair events. The parental sequence is shown as double-stranded DNA, whereas only the upper strand is shown for the sequenced DNA junctions. The *I-SceI* restriction site is in dark blue (with black triangles indicating the cut sites), the nucleotide insertions in red, and the deletions in bold green. Large deletions are noted as numbers of deleted nucleotides. The number of events found is indicated in the right column.

Strain and condition	plasmid sequence	mutation	plasmid position of mutation (nt)	<i>I-SceI</i> CDS position of mutation (nt)	mutation effect
WT Log phase (28°C)	TGAACACC <b>A</b> AGTCTTTCAC	C> <b>A</b>	3057	487	CAG (Q163) > <b>AAG (K)</b>
	CCTGATCCCG <b>T</b> AGATGATGTAC	C> <b>T</b>	3219	649	CAG (Q217) > <b>TAG (STOP)</b>
	CAACAAAAACAAC <b>A</b> GATCATCTACAT	C> <b>A</b>	3151	581	CCG (P194) > <b>CAG (Q)</b>
$\Delta ku2$ Log phase (28°C)	GGGTCCGA <b>A</b> CTCTAAACTGCTGAAAGAATACAA <b>IS</b>	ISRm1 (~1.3 kb) insertion	2632	62	Protein interrupted by <b>IS</b> after Y20
	CAGGTATCCGGTCTGATCCCTGGG <b>IS</b>	ISRm1 (~1.3 kb) insertion	2692	122	Protein interrupted by <b>IS</b> after L40
	CCCAGACTTT-AAACACCAA	Deletion : <b>C</b>	2875	305	Frameshift after T101
	GAAGCAGGTATCG <b>C</b> CTGTGATCCCTGGG	<b>G&gt;C</b>	2680	110	GGT (G37) > <b>GCT (A)</b>
$\Delta ku1234$ Log phase (28°C)	GGTCTGATCCCTGG <b>A</b> TGATGCTTACATCC	<b>G&gt;T</b>	2692	122	GGT (Q41) > <b>GAT (D)</b>
	TACATGGACCACGTATGTCTG <b>IS</b>	ISRm1 (~1.3 kb) insertion	2783	213	Protein interrupted by <b>IS</b> after L71
	AAAAACAAACCGATCATCT <b>IS</b>	ISRm1 (~1.3 kb) insertion	3160	590	Protein interrupted by <b>IS</b> after I196
	ACATCCGTCTCTGTGAT <b>IS</b>	ISRm1 (~1.3 kb) insertion	2719	149	Protein interrupted by <b>IS</b> after D49
$\Delta ligD1234$ Log phase (28°C)	GATGCTTACAT <b>C</b> ACGTTCTCGTGA	Insertion : <b>A</b>	2706	136	Frameshift after I45
	AACTGAACATCGAACAGT <b>IS</b>	ISRm1 (~1.3 kb) insertion	2664	94	Protein interrupted by <b>IS</b> after Q31
WT Log phase (40°C)	TTCTATGTCTT <b>A</b> ACTGATCTTCTAC	C> <b>A</b>	3179	609	TAC (Y203) > <b>TAA (STOP)</b>
	GCAGGTATCGGTCTG <b>\Delta 80 bp</b> CATGGACCACGTATG	Deletion : <b>80 bp</b>	2685	115	Loss of 26 a.a. and frameshift after L38
	GGTGGTAAA <b>\Delta 17 bp</b> CTCTACCAAC	Deletion : <b>17 bp</b>	3009	439	Loss of 5 a.a. and frameshift after K146
$\Delta ku2$ Log phase (40°C)	AACTGAACATCGAA <b>T</b> AGTTCGAAGCAGG	C> <b>T</b>	2661	91	CAG (Q31) > <b>TAG (STOP)</b>
	GACTTTCAAACAC <b>\Delta 994 bp</b> TTCTCCAACGAGCCT	Deletion : <b>994 bp</b>	2883	313	Loss of last 129 a.a. after H104
	GAAGAAGTA <b>T</b> AATACCTGG	<b>G&gt;T</b>	3081	511	GAA (E170) > <b>TAA (STOP)</b>
	ACTGCGCCGGTTAA <b>\Delta 124 bp</b> GCTGATCGAACTGAA	Deletion : <b>124 bp</b>	2514	-56	124 bp deleted just downstream the transcription start site
	GGGGCGCCAG <b>A</b> ATTTCAAACACCAAG	C> <b>A</b>	2872	302	ACT (T101) > <b>AAT (N)</b>
	TACGATCAG <b>C</b> GGGTACTGT	T> <b>C</b>	2796	226	TGG (W76) > <b>CGG (R)</b>
$\Delta ku1234$ Log phase (40°C)	AAATGGGAT <b>T</b> GAACAAAA	C> <b>G</b>	3017	447	TAC (Y149) > <b>TAG (STOP)</b>
	GTACGATCAGT <b>G</b> TGTACTGTCCCC	<b>G&gt;T</b>	2798	228	TGG (W76) > <b>TGT (C)</b>
	GATGCTTACAT <b>C</b> ACGTTCTCGTGA	Insertion : <b>A</b>	2706	136	Frameshift after I45
$\Delta ligD1234$ Log phase (40°C)	TTCCA <b>A</b> CTGA <b>A</b> CTTTTACGTAAAAAT	<b>G&gt;T</b>	3124	554	TGT (C185) > <b>TTT (F)</b>
	AAAAACAAACCGATCATCTAC <b>IS</b>	ISRm1 (~1.3 kb) insertion	3162	592	Protein interrupted by <b>IS</b> after Y197
	ACTGAACAC <b>C</b> GAGTCTTCACTTT	C> <b>G</b>	3057	487	CAG (Q163) > <b>GAG (E)</b>
	AAAACCTACT <b>A</b> TATGCAGTTC	<b>G&gt;A</b>	2734	164	TGT (C55) > <b>TAT (Y)</b>

**Figure S4.** Sequence analysis of *I-SceI*-expressing plasmids extracted from clones showing an intact *I-SceI* restriction site. Competent cells of the *S. meliloti* strains CBT2173 (WT), CBT2175 ( $\Delta ku2$ ), CBT2177 ( $\Delta ku1234$ ) and CBT2496 ( $\Delta ligD1234$ ) carrying a *I-SceI* restriction site in the chromosome were prepared either at 28°C (blue) or 40°C (red) and were transformed with the *I-SceI*-expressing plasmid pLS273-25. Plasmid DNA was extracted from 29 colonies carrying an intact *I-SceI* site originating from the different strains and conditions, and the sequence of the *I-SceI* meganuclease encoding gene of these plasmids was determined. The figure shows the mutations found in red.



insertion in the expected orientation		<i>spec<sup>R</sup></i> insert		number
	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	
	ACCCCTAGGATCCC <b>TATTA</b> AACCTCGAA	//	GGGTT <b>TATT</b> GTCCCATTACCTAGCGGC	
WT (Log phase 28°C)	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	2
	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	1
			total:	3
WT (Log phase 40°C)	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	14
	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	1
	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> <b>Δ146</b> ATCAT	1
	TGGGGATCCTAGGG <b>ATAA</b> <b>Δ262</b> GCC	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	1
			total:	17
$\Delta ku2$ (Log phase 40°C)	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	2
	TGGGGATCCTAGGG <b>ATAA</b> TTGGAGCTT	//	CCCAA <b>ATAA</b> CAGGGTAATGGATCGCCG	1
			total:	3
insertion in the opposite orientation		<i>spec<sup>R</sup></i> insert		number
	TGGGGATCCTAGGG <b>ATAA</b> TTGGG	//	TCCAA <b>TATAT</b> CAGGGTAATGGATCGCCG	
	ACCCCTAGGATCCC <b>ATAA</b> AACCC	//	AGGTT <b>TATT</b> GTCCCATTACCTAGCGGC	
WT (Log phase 28°C)	TGGGGATCCTAGGG <b>ATAA</b> <b>TAT</b> TTGGG	//	TCCAA <b>TATATATAA</b> CAGGGTAATGGATCGCCG	1
			total:	1
WT (Log phase 40°C)	TGGGGATCCTAGGG <b>ATAA</b> <b>TAT</b> TTGGG	//	TCCAA <b>TATATATAA</b> CAGGGTAATGGATCGCCG	1
	TGGGGATCCTAGGG <b>ATAA</b> <b>TAT</b> TTGGG	//	TCCAA <b>TATATATAA</b> CAGGGTAATGGATCGCCG	1
	TGGGGATCCTAGGG <b>ATAA</b> <b>TAT</b> TTGGG	//	TCCAA <b>TATATATAA</b> CAGGGTAATGGATCGCCG	1
	TGGGGATCCTAGGG <b>ATAA</b> <b>TAT</b> TTGGG	//	TCT <b>Δ252</b> <b>ATAA</b> CAGGGTAATGGATCGCCG	1
			total:	5

**Figure S5.** Sequence of junctions of insertion events at the *rhaS* locus carrying a *I-SceI* restriction site. Competent cells of the *S. meliloti* strains CBT2173 (WT) and CBT2175 ( $\Delta ku2$ ) carrying a *I-SceI* restriction site in the chromosome and the *I-SceI*-expressing plasmid pLS273-25\* under the control of a cumate inducible promoter were prepared either at 28°C (blue) or 40°C (red) in the presence of cumate. Cells were transformed with a linear DNA cassette carrying a Spec resistance gene flanked by *I-SceI*-compatible restriction sites generated with *Bst*XI (dark blue). This figure shows the DNA sequence of junctions in transformants having inserted the Spec resistance cassette at the chromosomal *I-SceI* site.

Insertions in the expected (upper part) or opposite (lower part) orientation are shown. Parental sequences are indicated as double-stranded DNA, whereas only the upper strand is shown for the sequenced DNA junctions. The 3' compatible protruding ends are shown in bold and the non-compatible 3' ends of inverted insertion events are in bold underlined.

Deleted nucleotides are indicated in green and large deletions are noted as numbers of deleted nucleotides. The number of events found is indicated in the right column.

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