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-BamHI* and *BamHI-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 *BglII* digestion and subcloned into the pJQ200mp19-derivative pLS272 (see construction details below) in *BamHI*. *ku2* was extracted from pJET1.2 by *XhoI-XbaI* digestions and subcloned into pLS272 in *NsiI-AvrII*. Before *XbaI* or *AvrII* digestions, *XhoI* and *NsiI* 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 Δ*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-cutt 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 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 µM 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 β-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 TetR 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 GmR gene from pBBR1MCS-5).

DNA integration assays
The DNA fragment conferring spectinomycin resistance was amplified from pHP45-Ω 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 |
|-------------------|-------------|---------------------|
| **Strains**        |             |                     |
| *Sinorhizobium meliloti* |             |                     |
| GMI11495          | wild-type strain (Sm<sup>β</sup>), Rm2011 background | (2, 3) |
| CBT1809           | GMI11495 ∆ku1 (SMa0426) | This work |
| CBT1810           | GMI11495 ∆ku2 (SMb20686) | This work |
| CBT1823           | GMI11495 ∆ku34 (SMb21406-21407) | This work |
| CBT1892           | GMI11495 ∆ku12 | This work |
| CBT1893           | GMI11495 ∆ku234 | This work |
| CBT1960           | GMI11495 ∆ku123 | This work |
| CBT1961           | GMI11495 ∆ku124 | This work |
| CBT1899           | GMI11495 ∆ku1234 | (4) |
| CBT1938           | GMI11495 ∆recA | (4) |
| CBT1943           | GMI11495 ∆recA ∆ku1234 | (4) |
| CBT2183           | GMI11495 ∆ku2 rhaS::ku2 | This work |
| CBT2381           | GMI11495 ∆ku123 rhaS::ku3 | This work |
| CBT2383           | GMI11495 ∆ku124 rhaS::ku4 | This work |
| CBT2385           | GMI11495 ∆ku1234 rhaS::ku34 | This work |
| CBT2382           | GMI11495 ∆ku1234 rhaS::ku3 | This work |
| CBT2384           | GMI11495 ∆ku1234 rhaS::ku4 | This work |
| CBT1813           | GMI11495 ∆ligD1 (SMa0414-0417) | This work |
| CBT2162           | GMI11495 ∆ligD2 (SMb20685) | This work |
| CBT1811           | GMI11495 ∆ku3 (SMb21406) | This work |
| CBT1815           | GMI11495 ∆ligD3 (SMb21044) | This work |
| CBT1816           | GMI11495 ∆ligD4 (SMc03959) | This work |
| CBT2158           | GMI11495 ∆ligD134 | This work |
| CBT2180           | GMI11495 ∆ligD234 | This work |
| CBT2164           | GMI11495 ∆ligD1234 | This work |
| CBT2172           | GMI11495 ∆ligD2 rhaS::ligD2 | This work |
| CBT2000           | GMI11495 ∆ku12 ∆ligD2 | This work |
| CBT1962           | GMI11495 ∆ku12 ∆ligD4 | This work |
| CBT2005           | GMI11495 ∆ku12 ∆ligD24 | This work |
| CBT2082           | GMI11495 ∆ku12 ∆ligD234 | This work |
| CBT2120           | GMI11495 ∆ku12 ∆ligD1234 | This work |
| CBT2173           | GMI11495 rhaS::I-SceI | This work |
| CBT2175           | GMI11495 ∆ku2 rhaS::I-SceI | This work |
| CBT2177           | GMI11495 ∆ku1234 rhaS::I-SceI | This work |
| CBT2496           | GMI11495 ∆ligD1234 rhaS::I-SceI | This work |
| CBT907            | GMI11495 rpoE2::hph | (3) |
| CBT2003           | GMI11495 ∆ku12 rpoE2::hph | This work |
| **Escherichia coli** |             |                     |
| DH5α              | F<sup>·</sup> Φ80<sub>LacZAM15</sub> Δ(lacZYA-argF) U169 recA1 endA1 thi-1 gyrA96 relA1 λ<sup>−</sup> | Invitrogen |
|                  | hsdR17(κ<sup>−</sup>, m<sup>−</sup>*) phoA supE44 thi-1 gyrA96 relA1 λ<sup>−</sup> |           |
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**: Vector (Spec<sup>R</sup>)
  - (7)
- **pCZ962**: Cloning vector (Tet<sup>R</sup> Amp<sup>R</sup>)
  - (8)
- **pDAI-SceI**: I-SceI constitutive expression vector (Tet<sup>R</sup>)
  - (9)
- **pQF**: Cumate inducible expression vector (Tet<sup>R</sup>)
  - (10)
- **pDP39**: pJQ200mp19 derivative for *ku1* deletion
  - this work
- **pDP40**: pJQ200mp19 derivative for *ku2* deletion
  - this work
- **pDP41**: pJQ200mp19 derivative for *ku3* deletion
  - this work
- **pDP42**: pJQ200mp19 derivative for *ku4* deletion
  - this work
- **pDP56**: pJQ200mp19 derivative for *ku34* deletion
  - this work
- **pDP43**: pJQ200mp19 derivative for *ligD1* deletion
  - this work
- **pDP113**: pJQ200mp19 derivative for *ligD2* deletion
  - this work
- **pDP45**: pJQ200mp19 derivative for *ligD3* deletion
  - this work
- **pDP46**: pJQ200mp19 derivative for *ligD4* deletion
  - this work
- **pDP55**: pJQ200mp19 derivative for *ligD2ku2* deletion
  - this work
- **pDP52**: pJQ200mp19 derivative for *recA* deletion
  - this work
- **pLS272-1**: pJQ200mp19 derivative for *I-SceI* insertion at the rhaS locus
  - this work
- **pLS282-3**: pJQ200mp19 derivative for 2 *I-SceI* insertion at the rhaS locus
  - this work
- **pDP115**: pLS272-1 derivative for *ligD2* insertion at the rhaS locus
  - this work
- **pDP116**: pLS272-1 derivative for *ku2* insertion at the rhaS locus
  - this work
- **pLS289-9**: pLS282-3 derivative for *ku3* insertion at the rhaS locus
  - this work
- **pLS290-13**: pLS282-3 derivative for *ku4* insertion at the rhaS locus
  - this work
- **pLS294-8**: pLS282-3 derivative for *ku34* insertion at the rhaS locus
  - this work
- **pDP66**: pCZ962 derivative for measure of *ku34* promoter activity
  - this work
- **pDP67**: pCZ962 derivative for measure of *ligD4* promoter activity
  - this work
- **pDP68**: pCZ962 derivative for measure of *ku1* promoter activity
  - this work
- **pDP69**: pCZ962 derivative for measure of *ku2* promoter activity
  - this work
- **pDP70**: pCZ962 derivative for measure of *ligD2* promoter activity
  - this work
- **pDP92**: pCZ962 derivative for measure of *ligD1* promoter activity
  - this work
- **pDP93**: pCZ962 derivative for measure of *ligD3* promoter activity
  - this work
- **pDP58**: pGEM-T derivative with *sacB*
  - this work
- **pDP62**: pBBR1MCS-5 with complete *lacZ* gene
  - this work
- **pDP63**: pDP62 with *sacB* inside *lacZ* in BamHI site
  - this work
- **pDP64**: pDP62 with *sacB* inside *lacZ* in SmaI site
  - this work
- **pDP65**: pDP62 with *sacB* inside *lacZ* in PstI 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-SceI* constitutive expression
  - this work
- **pLS273-25**<sup>+</sup>: pLS273-25 derivative for *I-SceI* cumate inducible expression
  - this work
- **pLS278-9**: pBBR1MCS-5 derivative with Spec<sup>R</sup> cassette
  - this work
Table S2: Oligonucleotides used in this study.

| Name       | Sequence (5'→3') | Target       |
|------------|------------------|--------------|
| OCB1284    | GATCCGGAATGGCACTCCG | fw ku1 upstream |
| OCB1285    | GAGCTCGAGGCTCTTGCTG | rev ku1 upstream |
| OCB1286    | GTCGACAAATGGGACCTCCG | fw ku1 downstream |
| OCB1287    | GATCCGCTCTTGGGAAATCGAGC | rev ku1 downstream |
| OCB1272    | GTCACTAAAGACATCTGCTGGC | fw ku2 upstream |
| OCB1273    | GATTCCTGACATTGTATTCTCCCTACG | rev ku2 upstream |
| OCB1274    | GATCCGCAAGAAGAAAGCGTATGG | fw ku2 downstream |
| OCB1275    | GAGCTCGAGGCGGCTTTCCGGAGC | rev ku2 downstream |
| OCB1260    | GTCGACACGCGTCAGGCGGACC | fw ku3 upstream |
| OCB1261    | GATCCTGCGATGCTCAGTTCTG | rev ku3 upstream |
| OCB1262    | GATCCGCGCAATAGGGAAGTGAACG | fw ku3 downstream |
| OCB1263    | GAGCTCCGACACGGTTCTAGCATCA | rev ku3 downstream |
| OCB1264    | GTCACTACAGGCGGCGAATGCCC | fw ku4 upstream |
| OCB1265    | GATCCGCGCATCTGCTCGCC | rev ku4 upstream |
| OCB1266    | GATCTAAAGAGGAGGATGCC | fw ku4 downstream |
| OCB1267    | GAGCTCTTGGGAGGCGGATGCC | rev ku4 downstream |
| OCB1292    | GAGCTCCAGGAGGCGGCGAATGCCC | fw ligD1 upstream |
| OCB1293    | GATCCGCGCAATAGGGAAGTGAACG | rev ligD1 upstream |
| OCB1294    | GATCCGCGCATCTGCTCGCC | fw ligD1 downstream |
| OCB1295    | GTCACTACAGGCGGCGAATGCCC | rev ligD1 downstream |
| OCB1268    | GAGCTCTCAGGCCGACTCCGGCGAAGC | fw ligD2 upstream |
| OCB1269    | GATCCGCGCATCGCCGCGCAAGC | rev ligD2 upstream |
| OCB1252    | GATCCGCGCAAGAAGATCGATGCC | fw ligD2 downstream |
| OCB1256    | GTCACTACAGGCGGCGAATGCCC | rev ligD2 downstream |
| OCB1280    | GATCCGCGCATCGCCGCGCAAGC | fw ligD3 upstream |
| OCB1281    | GAGCTCGACAGGAGGCGGCGAATGCCC | rev ligD3 upstream |
| OCB1282    | GTCACTACAGGCGGCGAATGCCC | rev ligD3 downstream |
| OCB1283    | GATCCGCGCATCTGCTCCGGG | rev ligD3 downstream |
| OCB1276    | CTCTTGTGACACGAGATGCC | fw ligD4 upstream |
| OCB1277    | GATCCGCGCATCGCCGCGCAAGC | rev ligD4 upstream |
| OCB1278    | GATCCGCGCATCGCCGCGCAAGC | fw ligD4 downstream |
| OCB1279    | GAGCTCCGACAGGAGGCGGCGAATGCCC | rev ligD4 downstream |
| OCB1302    | GAGCTCCGACAGGAGGCGGCGAATGCCC | fw recA upstream |
| OCB1303    | GATCCGACATCGTATCTCGGGAAGAACC | rev recA upstream |
| OCB1304    | GATCCGACATCGTATCTCGGGAAGAACC | rev recA downstream |
| OCB1305    | GTCACTACAGGCGGCGAATGCCC | rev recA downstream |

Screening of genomic deletions or insertions

| OCB1321    | GATGCTGGGAGATGATCCC | fw screening of Δku1 |
| OCB1322    | GCCACTGCGATGACGTCG | rev screening of Δku1 |
| OCB1323    | CGCAAGCTGCTCGGCAAGC | fw screening of Δku2 |
| OCB1324    | GAGCGAGAGATCGTTCCGGC | rev screening of Δku2 |
OCB1325  TTCTGACGAGCGTGATCGC  fw screening of Δku34
OCB1328  CTGCCGTCTGCTCCATGC  rev screening of Δku34
OCB1329  ACTGCGAATATCAGTCACC  fw screening of ΔligD1
OCB1330  GCCTCTGCTTGGCGTCC  rev screening of ΔligD1
OCB1331  GTGCCTGACGCGATTC  fw screening of ΔligD2
OCB1332  GCATCTGCTGCTCCATGC  rev screening of ΔligD2
OCB1333  CTGCTGACGCGATTC  fw screening of ΔligD3
OCB1334  CTGCCTGACGCGATTC  rev screening of ΔligD3
OCB1335  CCATCTGCTGCTCCATGC  fw screening of ΔligD4
OCB1336  GTGCCTGACGCGATTC  rev screening of ΔligD4
OCB1339  TGACGAGCAGACGCTTGGC  fw screening of ΔrecA
OCB1340  CACACCGGCACTCTCCG  rev screening of ΔrecA
OCB1347  TCTAGA  for ku2 complementation
OCB1414  CCATGG  for tetA tetR (pCZ962)
OCB1415  AGATCT  for tetA tetR (pCZ962)
OCB1417  GAGCTCCGATCGTACATGGG  fw sacB (pJQ200mp19)
OCB1418  ACCGGTACGCTGAGTGCAACATGGAAAATCG  rec sacB (pJQ200mp19)
OCB1431  GAGCTCAAGTCTCGAATCATGATGGG  fw 5’ rhaS (I-SceI addition)
OCB1432  GGATCCTAGGATCAGCGAGCTGCATGGG  rev 5’ rhaS (I-SceI addition)
OCB1433  GGATCCTAGGATCAGCGAGCTGCATGGG  rev 5’ rhaS (I-SceI addition)
OCB1434  GTGCACAGAAGTGCTGCTCCATGC  rev 3’ rhaS (I-SceI addition)
OCB1435  GGATCCTAGGATCGTACATGGG  fw spec^8 BstXII DNA end (pHP45-Ω)
OCB1436  GGATCCTAGGATCGTACATGGG  rev spec^8 BstXII DNA end (pHP45-Ω)
OCB1437  GATCCATATGCATAGCTAGTCCG  fw linker mcs + I-SceI addition
OCB1438  CTAGACTCGAGTAGGATAACATGGGAATCG  (pLS272-1 BamHI site)
| OCB1582 | GATCATTACCTGTTATCCCTACTCGAGT | rev linker mcs + I-SceI addition (pLS272-1 BamHI site) |
|---------|-----------------------------|-----------------------------------------------------|
|         | CTAGACCCGGGACTAGTCAATTGCATATG |                                                     |

Screening of NHEJ repair infidelity

| M13 rev | GGAAACAGCTATGACCAT | Universal primer |
|---------|-------------------|------------------|
| OCB1043 | AAGGGGGATGTGCTGCAAGG | pBBR1MCS-5 and derivatives (upstream multiple cloning site) |
| OCB1178 | CGTGCCCTTCATCCGTTCC | pBBR1MCS-5 and derivatives (downstream multiple cloning site) |

*Restriction sites are indicated in bold letters. I-SceI and BstXI sites are underlined.*
Table S3: Multiple mutant constructions.

| Strains   | Description                                      |
|-----------|--------------------------------------------------|
| CBT1809   | *ku1* deletion in GMI11495 (WT)                  |
| CBT1810   | *ku2* deletion in GMI11495 (WT)                  |
| CBT1813   | *ligD1* deletion in GMI11495 (WT)                |
| CBT1815   | *ligD3* deletion in GMI11495 (WT)                |
| CBT1816   | *ligD4* deletion in GMI11495 (WT)                |
| CBT1822   | *ligD2* and *ku2* deletion in GMI11495 (WT)      |
| CBT1823   | *ku34* deletion in GMI11495 (WT)                 |
| CBT1892   | *ku1* deletion in CBT1810                        |
| CBT1893   | *ku34* deletion in CBT1810                       |
| CBT1899   | *ku1* deletion in CBT1893                        |
| CBT1938   | *recA* deletion in GMI11495                      |
| CBT1943   | *recA* deletion in CBT1899                       |
| CBT1946   | *ku4* deletion in CBT1810                        |
| CBT1957   | *ku3* deletion in CBT1810                        |
| CBT1960   | *ku1* deletion in CBT1957                        |
| CBT1961   | *ku1* deletion in CBT1946                        |
| CBT1962   | *ligD4* deletion in CBT1892                      |
| CBT1997   | *ku2* deletion in CBT907 (*rpoE2::hph*)          |
| CBT2000   | *ku1* deletion in CBT1822                        |
| CBT2003   | *ku1* deletion in CBT1997                        |
| CBT2005   | *ligD4* deletion in CBT2000                      |
| CBT2082   | *ligD3* deletion in CBT2005                      |
| CBT2119   | *ligD1* deletion in CBT1815                      |
| CBT2120   | *ligD1* deletion in CBT2082                      |
| CBT2158   | *ligD4* deletion on CBT2119                      |
| CBT2162   | *ligD2* deletion in GMI11495 (WT)                |
| CBT2163   | *ligD2* deletion in CBT1816                      |
| CBT2164   | *ligD2* deletion in CBT2158                      |
| CBT2180   | *ligD3* 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 (BamHI) | 38              | 40.52   | 7   | 30  | <0.0001 |
| Fig.1A | Blunt end (SmaI)* | 27              | 3.831   | 4   | 22  | 0.0165  |
| Fig.1A | 3' end (PstI)  | 43              | 8.068   | 7   | 35  | <0.0001 |
| Fig.1B | 5' end (BamHI) | 24              | 64.42   | 4   | 19  | <0.0001 |
| Fig.1B | Blunt end (SmaI)* | 19              | 1.437   | 2   | 16  | 0.2668  |
| Fig.1B | 3' end (PstI)  | 26              | 5.24    | 4   | 21  | 0.0044  |
| Fig.2A | 5' end (BamHI) | 40              | 71.58   | 6   | 33  | <0.0001 |
| Fig.2A | Blunt end (SmaI)* | 32              | 39.16   | 4   | 27  | <0.0001 |
| Fig.2A | 3' end (PstI)  | 45              | 15.59   | 6   | 38  | <0.0001 |
| Fig.2B | 5' end (BamHI) | 67              | 110.2   | 13  | 53  | <0.0001 |
| Fig.2B | Blunt end (SmaI)* | 45              | 29.07   | 8   | 36  | <0.0001 |
| Fig.2B | 3' end (PstI)  | 70              | 35.21   | 13  | 56  | <0.0001 |
| Fig.3A | 5' end (BamHI) | 25              | 191.7   | 6   | 18  | <0.0001 |
| Fig.3A | Blunt end (SmaI)* | 16              | 11.29   | 4   | 11  | 0.0007  |
| Fig.3A | 3' end (PstI)  | 25              | 50.2    | 6   | 18  | <0.0001 |
| Fig.3B | 5' end (BamHI) | 25              | 37.55   | 5   | 19  | <0.0001 |
| Fig.3B | Blunt end (SmaI)* | 18              | 39.55   | 3   | 14  | <0.0001 |
| Fig.3B | 3' end (PstI)  | 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 (BamHI) | 27              | 33.91   | 3   | 23  | <0.0001 |
| Fig.4C | Blunt end (SmaI)* | 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 |
|----------------------|--------------------------|----------------------------|
|                      | BamHI  | SmaI  | PstI  | BamHI  | SmaI  | PstI  |
| **Log phase 28°C**   |        |       |       |        |       |       |
| WT                   | 94.0   | 78.5  | 98.5  | 6578   | 177   | 6894  |
| ΔrecA                | 91.5   | 83.3  | 98.6  | 199    | 6     | 138   |
| Δku1                 | 86.4   | 83.3  | 98.8  | 1481   | 24    | 1212  |
| Δku2                 | 100.0  | NA    | 100.0 | 26     | 0     | 415   |
| Δku34                | 88.7   | 72.4  | 97.2  | 2308   | 29    | 1153  |
| Δku1234              | 100.0  | NA    | 100.0 | 20     | 0     | 380   |
| Δku1234 ΔrecA        | 100.0  | NA    | 100.0 | 93     | 0     | 170   |
| ΔligD2               | 100.0  | NA    | 100.0 | 22     | 0     | 640   |
| ΔligD134             | 92.0   | 88.2  | 98.4  | 1391   | 17    | 610   |
| ΔligD1234            | 100.0  | NA    | 100.0 | 20     | 0     | 524   |
| **Stationary phase** |        |       |       |        |       |       |
| WT                   | 89.2   | 73.0  | 98.5  | 21341  | 1562  | 20063 |
| Δku1                 | 82.1   | 71.1  | 99.2  | 8244   | 646   | 11728 |
| Δku2                 | 95.1   | 67.8  | 97.0  | 1051   | 270   | 908   |
| Δku34                | 83.2   | 58.8  | 89.3  | 7171   | 719   | 2430  |
| Δku1234              | 100.0  | NA    | 98.8  | 55     | 0     | 2550  |
| Δku1234              | 100.0  | NA    | 100.0 | 58     | 0     | 727   |
| **Log phase 40°C**   |        |       |       |        |       |       |
| WT                   | 92.4   | 79.5  | 99.0  | 36035  | 2475  | 55006 |
| Δku1                 | 71.8   | 72.4  | 94.9  | 2050   | 4176  | 3702  |
| Δku2                 | 66.2   | 67.7  | 90.4  | 130    | 155   | 539   |
| Δku12                | 87.1   | 69.3  | 83.7  | 3035   | 940   | 2881  |
| Δku34                | 95.5   | 79.2  | 98.1  | 32078  | 1606  | 40628 |
| Δku1234              | 100.0  | NA    | 99.5  | 8      | 0     | 367   |
| ΔligD1234            | 100.0  | NA    | 100.0 | 124    | 0     | 2050  |
| Δku12 ΔligD2         | 74.5   | 63.4  | 80.4  | 987    | 1033  | 1400  |
| Δku12 ΔligD4         | 98.2   | 36.8  | 98.4  | 739    | 19    | 624   |
| Δku12 ΔligD24        | 100.0  | 0.0   | 99.9  | 19     | 56    | 950   |
| Δku12 ΔligD234       | 100.0  | NA    | 100.0 | 10     | 0     | 165   |
| Δku12 ΔligD1234      | 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 | BamHI | P value | PstI | P value |
|-----------------------|-------|---------|------|---------|
|                       | Blue (Lac⁺) | White (Lac⁻) |     | Blue (Lac⁺) | White (Lac⁻) |     |
|                       |         |         | P value |         |         |         |
| **Log phase 28°C**    |       |         |       |       |       |         |
| WT                    | 6185  | 393     | 0.0098 | 6788  | 106    | <0.0001 |
| ∆ku2+∆ligD2+∆ku1234+∆ligD1234 | 88    | 0       |         | 1959  | 0       |         |
| **Stationary phase**  |       |         |       |       |       |         |
| WT                    | 19026 | 2315    | 0.0023 | 19769 | 294    | <0.0001 |
| ∆ku1234              | 58    | 0       |         | 727   | 0       |         |
| ∆ku2                 | 881   | 27      | 0.0014  |       |         |         |
| ∆ku234               | 2518  | 32      |         |       |         |         |
| ∆ku1234              | 881   | 27      | <0.0001 |       |         |         |
|                       | 727   | 0       |         |       |         |         |
| **Log phase 40°C**    |       |         |       |       |       |         |
| WT                    | 33305 | 2730    | <0.0001 | 54440 | 566    | <0.0001 |
| ∆ku1234+∆ligD1234    | 132   | 0       | <0.0001 | 2414  | 3       | <0.0001 |
| ∆ku2                 | 487   | 52      | <0.0001 |       |         |         |
| ∆ku1234              | 365   | 3       |         |       |         |         |
| ∆ku12                | 2411  | 470     | <0.0001 |       |         |         |
| ∆ku1234              | 365   | 2       |         |       |         |         |
| ∆ku12 ∆ligD2        | 1125  | 275     |         |       |         |         |
| ∆ku12 ∆ligD24+∆ku12 ∆ligD234+ | 1510 | 1 | <0.0001 |
| ∆ku12 ∆ligD1234     |       |         |         |       |         |         |
| ∆ku12 ∆ligD4        | 614   | 10      | <0.0001 |       |         |         |
| ∆ku12 ∆ligD24+∆ku12 ∆ligD234+ | 1510 | 1 | <0.0001 |
| ∆ku12 ∆ligD1234     |       |         |         |       |         |         |
**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.
| Restriction Enzyme | WT Log phase (28°C) | WT Stationary phase (28°C) | WT Log phase (40°C) | ku12 Log phase (40°C) |
|--------------------|---------------------|-----------------------------|--------------------|-----------------------|
| **BamHI**          |                     |                             |                    |                       |
| number             |                     |                             |                    |                       |
| Total              | 30                  |                             | 3                  |                       |
| GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 26  | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 28  | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 4 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 8 |
| large deletion     | 3                   |                             | 1                  |                       |
| total              | 30                  |                             | 7                  |                       |
| GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 |
| **Smal**           |                     |                             |                    |                       |
| number             |                     |                             |                    |                       |
| Total              | 12                  |                             | 2                  |                       |
| GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 8 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 2 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 2 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 0 |
| large deletion     | 1                   |                             | 1                  |                       |
| total              | 12                  |                             | 3                  |                       |
| GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 |
| **PstI**           |                     |                             |                    |                       |
| number             |                     |                             |                    |                       |
| Total              | 10                  |                             | 1                  |                       |
| GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 3 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 3 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 2 | GCGGCCGCTCTAGAATGTGGATCCCTCCGCTGCGAGAATTCCGATAT 1 |
| large deletion     | 1                   |                             | 1                  |                       |
| total              | 10                  |                             | 9                  |                       |

*Figure S2.* Infidelity of NHEJ during linear plasmid repair in *Sinorhizobium meliloti*. Linear plasmid DNA generated by restriction digest with either *BamHI* (5' overhang), *Smal* (blunt) or *PstI* (3' overhang) was used to transform WT (CBT707) and Δ*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- 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+ colonies. Sequencing of plasmid DNA from 51 randomly chosen blue (Lac+) 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%. 
Figure S3. Infidelity of NHEJ during repair of chromosomal double-strand breaks. Competent cells of the *S. meliloti* strains CBT2173 (WT) and CBT2175 (Δku2) carrying an *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 an *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-SceI CDS position of mutation (nt) | mutation effect |
|----------------------|------------------|----------|----------------------------------|--------------------------------------|----------------|
| WT                   | TGAACCGAGCTCTTCCAC | C>T      | 3607                            | 487                                  | CAG (Q163) > AAG (K) |
| Log phase (28°C)     | CGGTATGGCTAGTATGAC | C>A      | 3219                            | 649                                  | CAG (Q217) > TAG (STOP) |
| Δku2                 | GGGTCCGAACCTCCTAAACTGCCGAAGAATACAA IS | ISrm1 (~1.3 kb) insertion | 2662                           | 62                                   | Protein interrupted by IS after Y20 |
| Log phase (28°C)     | CAGGTATGGCTAGTATGAC | ISrm1 (~1.3 kb) insertion | 2692                           | 122                                  | Protein interrupted by IS after L40 |
| Δku1234              | GGGTCCGAACCTCCTAAACTGCCGAAGAATACAA IS | ISrm1 (~1.3 kb) insertion | 3160                           | 590                                  | Protein interrupted by IS after L71 |
| Log phase (28°C)     | CAGGTATGGCTAGTATGAC | ISrm1 (~1.3 kb) insertion | 2719                           | 149                                  | Protein interrupted by IS after I96 |
| ΔligD1234            | GGGTCCGAACCTCCTAAACTGCCGAAGAATACAA IS | ISrm1 (~1.3 kb) insertion | 2706                           | 136                                  | Frameshift after I45 |
| Log phase (28°C)     | GGGTCCGAACCTCCTAAACTGCCGAAGAATACAA IS | ISrm1 (~1.3 kb) insertion | 2664                           | 94                                   | Protein interrupted by IS after Q31 |

| WT                   | TTCTAGTGGCTTTACTGCTCTCAC | C>A      | 3179                            | 609                                  | TAC (Y203) > TAA (STOP) |
| Log phase (40°C)     | GCAAGTGATCGGTCTGCG Δ80 bp CATGGCACAAGCTATG | Deletion : 80 bp | 2685                           | 115                                  | Loss of 26 a.a. and frameshift after L38 |
| Δku2                 | AACTGAAACTGGAAGTTGCTGACAGAGGG | Deletion : 994 bp | 2883                           | 313                                  | Loss of last 129 a.a. after H104 |
| Log phase (40°C)     | AACTGAAACTGGAAGTTGCTGACAGAGGG | Deletion : 994 bp | 3081                           | 511                                  | GAA (E170) > TAA (STOP) |
| Δku1234              | AACTGAAACTGGAAGTTGCTGACAGAGGG | Deletion : 994 bp | 2514                           | -56                                  | 124 bp deleted just downstream the transcription start site |
| Log phase (40°C)     | GGGTGACGGGTTGTA Δ124 bp GCTGACTGAAACGGTAA | Deletion : 124 bp  | 2872                           | 302                                  | ACT (T101) > AAT (n) |
| ΔligD1234            | GGGTGACGGGTTGTA Δ124 bp GCTGACTGAAACGGTAA | Deletion : 124 bp  | 2796                           | 226                                  | TGG (W76) > CGG (R) |
| Log phase (40°C)     | GGGTGACGGGTTGTA Δ124 bp GCTGACTGAAACGGTAA | Deletion : 124 bp  | 3017                           | 447                                  | TAC (Y149) > TAA (STOP) |
| Δku1234              | GGGTGACGGGTTGTA Δ124 bp GCTGACTGAAACGGTAA | Deletion : 124 bp  | 2798                           | 228                                  | TGG (W76) > TGT (c) |
| Log phase (40°C)     | GGGTGACGGGTTGTA Δ124 bp GCTGACTGAAACGGTAA | Deletion : 124 bp  | 2706                           | 136                                  | Frameshift after I45 |
| ΔligD1234            | TGGTATTGAAACAAAAAAA | C>G      | 3124                            | 554                                  | TGT (C185) > TTT (F) |
| Log phase (40°C)     | TGGTATTGAAACAAAAAAA | C>G      | 3162                            | 592                                  | Protein interrupted by IS after Y197 |
| ΔligD1234            | TGGTATTGAAACAAAAAAA | C>G      | 3057                            | 487                                  | CAG (Q163) > GAG (E) |

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 (Δku2), CBT2177 (Δku1234) and CBT2496 (ΔligD1234) carrying an 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.
**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 (Δku2) 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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