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Multiple Ku orthologues mediate DNA non-homologous end-joining in the free-living form and during chronic infection of *Sinorhizobium meliloti*

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Summary

The bacterial non-homologous end-joining (NHEJ) apparatus is a two-component system that uses Ku and LigD to repair DNA double-strand breaks. Although the reaction mechanism has been extensively studied, much less is known about the physiological role of bacterial NHEJ. Recent studies suggest that NHEJ acts under conditions where DNA replication is reduced or absent (such as in a spore or stationary phase). Interestingly, genes encoding Ku and LigD have been identified in a wide range of bacteria that can chronically infect eukaryotic hosts. Strikingly, *Sinorhizobium meliloti*, an intracellular symbiont of legume plants, carries four genes encoding Ku homologues (*sku1* to *sku4*). Deletion analysis of the *sku* genes indicated that all Ku homologues are functional. One of these genes, *sku2*, is strongly expressed in free-living cells, as well as in bacteroid cells residing inside of the host plant. To visualize the NHEJ apparatus *in vivo*, SKu2 protein was fused to yellow fluorescent protein (YFP). Ionizing radiation (IR) induced focus formation of SKu2-YFP in free-living cells in a dosage-dependent manner. Moreover, SKu2-YFP foci formed in response to IR in non-dividing bacteroids, indicating that NHEJ system is functional even during the chronic infection phase of symbiosis.

Introduction

DNA double-strand breaks (DSBs) pose a major threat to the genome and must be repaired properly to preserve genomic integrity. In higher eukaryotes, DSBs are predominately repaired by non-homologous end-joining (NHEJ), in which the two ends of a DSB are directly rejoined with little or no requirement for sequence homology (Hefferin and Tomkinson, 2005). Recently, an evolutionary-related NHEJ pathway was identified in many bacterial species (Weller *et al.*, 2002; Della *et al.*, 2004; Bowater and Doherty, 2006; Pitcher *et al.*, 2007a). In bacteria, NHEJ is mediated by the DNA end-binding Ku protein and LigD, a multifunctional

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Supplementary material

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DNA repair enzyme (Weller *et al.*, 2002; Della *et al.*, 2004). Ku forms a stable homo-dimer in solution and preferentially binds to the linear, double-stranded DNA *in vitro* (Weller *et al.*, 2002). LigD proteins often contain an ATP-dependent DNA ligase catalytic domain, a DNA polymerase domain and an exonuclease domain (Weller *et al.*, 2002; Pitcher *et al.*, 2005; 2007b; Zhu *et al.*, 2005; 2006; Zhu and Shuman, 2005a,b; 2006; Akey *et al.*, 2006; Yakovleva and Shuman, 2006). LigD therefore possesses most of the activities required to remodel the damaged DNA ends (filling in gaps and removing non-complementary extensions) and ultimately closing the nicks. Ku binds to the polymerase domain of LigD and stimulates the polymerase, exonuclease and ligase activities *in vitro* (Weller *et al.*, 2002; Pitcher *et al.*, 2005). These *in vitro* results suggest that, *in vivo*, Ku and LigD form a two-component DSB repair complex in which the Ku homo-dimer recruits LigD to a DSB and modulates the end-processing activity of LigD. Nucleotide sequence analysis of the repaired junctions often shows mutations, which results from the LigD-dependent end processing (Gong *et al.*, 2005; Stephanou *et al.*, 2007). Thus, NHEJ is referred to as an error-prone repair pathway (Gong *et al.*, 2005).

Under laboratory conditions, bacteria generally replicate DNA during most of their life cycle. Thus, sister chromosomes are available for homologous recombination (HR), an error-free pathway of DSB repair that relies on strand exchange between the broken DNA and its intact sister chromosome (Friedberg *et al.*, 2006). In contrast, cells of higher eukaryotes are usually in the non-replicating states (G₁ phase of the cell cycle) or terminally differentiated (G₀ phase). Repair of DSBs depends solely on NHEJ in such non-replicating cells, as a homologous chromosome is not available for HR. Analogously, bacterial NHEJ likely functions during the cell's slow- or non-replicating stage: In *Bacillus subtilis*, the expression of *ykoU* and *ykoV* (encoding the LigD and Ku proteins respectively) is under the control of σ^G , the forespore-specific sigma factor for RNAPolymerase, and is induced during the forespore development (Wang *et al.*, 2006). The GFP-tagged Ku (YkoV) protein localizes to the nucleoid in newly germinating spores, whereas it is distributed equally in the spore core before germination and disappears in the later stage of germination. As ATP is not available in the dormant spores, it has been suggested that the NHEJ apparatus is accumulated during sporulation, stored in spores, and functions specifically during spore germination. Disruptions of *ykoU* and/or *ykoV* significantly sensitize spores to DSB-inducing treatments, such as ionizing radiation (IR), UV, dry heat and extreme desiccation (Moeller *et al.*, 2007). In *Mycobacterium smegmatis*, disruption of NHEJ genes sensitizes stationary-phase cultures, but not logarithmic cultures, to IR and desiccation (Pitcher *et al.*, 2007c; Stephanou *et al.*, 2007).

Interestingly, genes encoding the NHEJ apparatus have been identified in a wide range of pathogens (such as *Agrobacterium tumefaciens*, *Bacillus anthracis*, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*) and symbionts (such as *Sinorhizobium meliloti* and other rhizobia) that can chronically infect their eukaryotic hosts (Bowater and Doherty, 2006). Entry into the chronic phase of infection is generally accompanied by a marked reduction or absence of bacterial DNA replication (Brown and Smith, 2001; Muñoz-Elías *et al.*, 2005; Furukawa *et al.*, 2006; Mergaert *et al.*, 2006). It therefore seems plausible that NHEJ may function during chronic infection in these bacteria.

Sinorhizobium meliloti, a member of *Rhizobiales*, represents a particularly interesting model organism in this respect, because the symbiotic relationship with the plant host is based on a chronic intracellular infection (Jones *et al.*, 2007). *S. meliloti* induces the formation of a highly specialized organ, the nodule, on the root of the host plant, and intracellularly colonizes the tissue of the nodule (Broughton *et al.*, 2000; Perret *et al.*, 2000). In soil or liquid culture, *S. meliloti* is a rod-shaped cell of 1–2 μm , the so-called 'free-living' form. Upon infecting the cytoplasm of host cells, bacteria differentiate into bacteroids, the specialized symbiotic form which enzymatically reduce atmospheric nitrogen, thereby providing a source of fixed nitrogen

for the host (Kaminski *et al.*, 1998; Prell and Poole, 2006). During the bacteroid differentiation, DNA replication is repeated several times without cell division, resulting in formation of elongated (5–10 μ m) cells containing up to 24 copies of their genome. Once fully differentiated, however, bacteroids lose their ability to resume growth and no further DNA replication occurs in these cells (Kobayashi *et al.*, 2001; Mergaert *et al.*, 2006). Thus, bacteroids are terminally differentiated, which is analogous to the G₀ phase of eukaryotic cells.

Strikingly, multiple copies of Ku have been detected in *S. meliloti* and all other rhizobia to date (Rocha *et al.*, 2005). The genome of *S. meliloti* strain Rm1021 carries four open reading frames (ORFs) encoding Ku homologues (see Fig. 1), whereas other bacteria possessing the NHEJ system typically encode a single Ku (Wilson *et al.*, 2003; Rocha *et al.*, 2005). Here, we report the *in vivo* analysis of the multiple NHEJ systems in *S. meliloti* strain Rm1021. Our results suggest that *S. meliloti* Ku proteins contribute to maintaining genomic stability in the free-living form and during chronic intracellular infection.

Results

S. meliloti Rm1021 possesses four functional homologues of Ku

Our examination of the sequenced *S. meliloti* Rm1021 genome revealed that it contained four ORFs encoding close homologues of bacterial Ku (*sku1*, *sku2*, *sku3* and *sku4*; Fig. 1). Five ORFs encoding LigD homologues were also identified (*ligD1L*, *ligD1P*, *ligD2*, *ligD3* and *ligD4*; Fig. 1). *ligD2* and *ligD4* encode proteins homologous to the *P. aeruginosa* LigD, in which the nuclease and polymerase domains are N-terminal and C-terminal extensions, respectively, of the ligase domain (Zhu and Shuman, 2005b). *LigD3* lacks the nuclease domain, similar to the YkoU protein of *B. subtilis* (Bowater and Doherty, 2006). *ligD1L* encodes a ligase domain with an N-terminal nuclease domain, whereas *ligD1P* encodes only a polymerase domain. Additionally, the *ligC* ORF encodes only the ligase domain of LigD, which is similar to the *M. smegmatis* LigC (Gong *et al.*, 2005). *sku1* and *sku2* are adjacent to *ligC* and *ligD2* respectively, while *sku3* and *sku4* are arranged tandemly and likely form an operon (the intergenic region between two genes is 14 bp). Interestingly, only *ligD4* is encoded in the chromosome. *sku1*, *ligD1L*, *ligD1P* and *ligC* are encoded on the symbiotic megaplasmid pSymA, while *sku2*, *sku3*, *sku4*, *ligD2* and *ligD3* are encoded on pSymB, a distribution that suggests a possible role for NHEJ during symbiosis.

To examine the function of Ku homologues in strain Rm1021, non-polar deletion mutants of the *sku* genes (Rm1021 Δ *sku1*, Rm1021 Δ *sku2*, Rm1021 Δ *sku3* and Rm1021 Δ *sku4*) were constructed. Saturated cultures of deletion mutants were irradiated with IR (0, 200, 400 and 600 Gy) and plated to measure viability. Irradiation of each *sku* deletion strain caused a significant decrease in viability compared with the wild type (Fig. 2A), indicating that all four *sku* genes encode functional orthologues of bacterial Ku protein. Interestingly, deletion of *sku2* had a more profound effect on IR sensitivity than deletion of any other *sku* genes (Fig. 2A). The IR sensitivities of strains containing deletions of multiple *sku* genes (Rm1021 Δ *sku13*, Rm1021 Δ *sku134* and Rm1021 Δ *sku1234*) were also determined (Fig. 2B). As expected, deletion of all four *sku* genes in the same background (Rm1021 Δ *sku1234*) resulted in an IR sensitivity greater than any of the single *sku* deletion strains (Fig. 2B). There was no significant difference between *sku* deletion mutants and the parental strain in the growth, viability or cell morphology under normal growth conditions (data not shown).

In vivo and *in planta* expression profiles of *sku* and other NHEJ genes

The existence of multiple NHEJ genes suggested that they might be differentially expressed under distinct physiological conditions, and thus might be specialized for different physiological roles. To address this model, we determined the expression of the *S. meliloti*

sku genes. The regions inferred to contain promoters of *sku1*, *sku2* and the *sku3sku4* operon were cloned into pMP220, a broad-host range transcriptional-*lacZ* reporter system (Spaink *et al.*, 1987; yielding plasmids pMP-*sku1*p, pMP-*sku2*p and pMP-*sku3*p respectively). The promoter region of *recA*, which encodes an essential protein for HR (Friedberg *et al.*, 2006), was also examined as an example of a SOS-regulated gene whose expression is induced by DNA damage (plasmid pMP-*recA*p).

For promoters of *sku1* and the *sku3sku4* operon, the regions upstream of *sku1* and *sku3* were examined (Fig. 1), because ORFs upstream of *sku1* and the *sku3* (SMa0429 and SMb21405) are distantly located (287 bp and 173 bp away from start codons of *sku1* and *sku3* respectively), and encode putative proteins that were not known to be involved in NHEJ. In the case of the *sku2* promoter, *sku2* is located 96 bp downstream of *ligD2* in the same orientation, suggesting that these genes might form an operon, which is transcribed from a promoter upstream of *ligD2*. To determine the operon structure of *ligD2* and *sku2*, the polar effect of the insertional disruption of *ligD2* on the transcription of *sku2* was examined. We replaced the genomic copy of *sku2* by *lacZ*, resulting in the strain Rm1021*sku2*Δ*lacZ*. The strain showed significantly higher β-galactosidase activity (1393 ± 21 units) than the parental strain (2.2 ± 0.3 units) 24 h after subculture. A polar mutant of *ligD2* was then created by inserting pJH104 (Ferguson *et al.*, 2005), an integration vector carrying a promoter-less *uidA* followed by a transcriptional terminator, into *ligD2* in the Rm1021*sku2*Δ*lacZ* background. More than 70% of the *lacZ* activity (1023 ± 27 units) was retained in the presence of the pJH104 insertion in *ligD2*. Thus, although the transcription of *sku2* is affected by the readthrough from the *ligD2* promoter, *sku2* is mainly transcribed by the promoter located in the intergenic region between *ligD2* and *sku2*. We therefore cloned the region directly upstream of *sku2* as the *sku2* promoter (Fig. 1).

Exponentially growing cells were inoculated into fresh culture media, and the β-galactosidase activities of Rm1021 strains harbouring these plasmids were monitored 1, 3, 6, 9 and 24 h after subculture (h) (Fig. 3A). Cells were in early- to mid-logarithmic growth phase ($^{1\text{cm}}\text{OD}_{600}$ of 0.3–0.6) from approximately 3 to 9 h and were in stationary phase ($^{1\text{cm}}\text{OD}_{600}$ of > 2.0) by 24 h (Fig. 2A).

In free-living conditions in rich medium, *sku2* had the strongest promoter (3370 ± 15 units, 24 h), whereas significantly lower activities were recorded for the promoters of *sku1* and the *sku3sku4* operon (50 ± 3 and 30 ± 7 units, respectively) (Fig. 2A). Interestingly, the *sku2* promoter activity was significantly enhanced at 24 h, by which time the cells were in stationary phase (a 3.3-fold higher activity than that of 1 h) (Fig. 3A). This observation suggested either that transcription of *sku2* is enhanced during stationary phase or that it is under the control of one of *S. meliloti*'s quorum-sensing systems. To examine the possible involvement of quorum-sensing in *sku2* regulation, the *sku2* promoter activity was assessed in conditioned media derived from stationary culture of strain Rm1021 or Rm1021 (pMP-*sku2*p). Exposure of log-phase cells to conditioned media did not affect the transcription of *sku2* (data not shown), indicating that quorum response is not involved in enhancing transcriptional activity of the *sku2* promoter.

In *S. meliloti* (Tapias and Barbé, 1999), as in many other bacteria (Friedberg *et al.*, 2006), expression of genes involved in HR pathway (such as *recA*) is induced by DNA damage in a process termed the SOS response, which is mediated by RecA and LexA proteins (Friedberg *et al.*, 2006). To examine the effect of DNA damage on the transcription of *sku* genes, mitomycin C (MMC), a well-characterized inducer of the SOS response (Kenyon and Walker, 1980), was added to cultures at 1 h (Fig. 3B). Transcription from the *recA* promoter was induced twofold by the addition of MMC, consistent with a previous study of *recA* regulation in *S. meliloti* (Riera *et al.*, 1994). In contrast, exposing cells to MMC did not affect the transcription of *sku2* or other *sku* genes. As expected, a disruption of the chromosomal *recA*⁺ gene abolished

the induction of the *recA* promoter by MMC; however, it did not alter the transcription of *sku2* (data not shown). Thus, in the free-living cells of strain Rm1021, expression of *sku2* is not controlled by the SOS response, but rather is growth phase-dependent.

To assess whether one or more of *sku* genes might be differentially expressed in nodules during symbiosis, the *sku* genes were transcriptionally fused to *uidA* by inserting pJH104 into *sku1*, *sku2*, *sku3* and *sku4*. In the free-living stage, strong β -glucuronidase activity was only detected in the case of the *sku2::uidA* fusion on X-gluc indicator plates (data not shown), confirming the results we obtained with the *sku* promoter-*lacZ* fusions (Fig. 3A and B). Strains carrying *sku::uidA* fusions elicited functional pink nodules on alfalfa, indicating that bacteria successfully infected nodule tissues. The nodules were sectioned and stained for β -glucuronidase activity (Fig. 3C–F). *S. meliloti* induces formation of ‘indeterminate’-type nodules with a persistent meristem (which is marked with asterisks in Fig. 3C–J) on the roots of host plants. The *sku2::uidA* fusion exhibited strong β -glucuronidase expression throughout the nodule (Fig. 3D), whereas expression of *sku3* and *sku4* fusions occurred in a limited region close to the meristematic zone of the nodule. The region of the nodule where *sku3* and *sku4* are expressed corresponds to the invasion zone (Jones *et al.*, 2007), where bacteria are being released into the plant cell and undergo the initial differentiation into bacteroids (Fig. 3E and F). The β -glucuronidase activity of the *sku3* fusion was stronger than that of the *sku4* fusion. As described above, *sku3* and *sku4* are likely in an operon which is transcribed from the promoter located upstream of *sku3*. The higher expression of the *sku3* fusion compared with the *sku4* fusion likely resulted from the pJH104 insertion being located closer to the promoter in Rm1021*sku3::pJH104*. We were unable to detect expression of the *sku1* fusion in alfalfa nodules (Fig. 3C).

To examine whether the various *ligD* genes were also differentially regulated, we constructed *uidA* fusions to the four *ligD* genes that contain a polymerase domain (*ligD1P*, *ligD2*, *ligD3* and *ligD4*). Free-living cells of the strain carrying the *ligD2::uidA* fusion exhibited β -glucuronidase activity on the X-gluc indicator plate at a level similar to the *sku2::uidA* fusion strain (data not shown). Moreover, the *ligD2::uidA* fusion and the *sku2::uidA* fusion shared virtually an identical expression profile in the nodule (Fig. 3D and H). The similar expression pattern of *sku2* and *ligD2*, and the fact that the two genes are adjacent, suggest that SKu2 and LigD2 function together in NHEJ. Similarly, the *ligD4::uidA* and the *sku3::uidA* fusions shared a similar pattern of expression specific to the invasion zone (Fig. 3E and J). We could not detect expression of *ligD1P* and *ligD3* fusions in alfalfa nodules (Fig. 3G and I).

Our observations indicate that each NHEJ gene is regulated in a temporally and spatially distinct manner. Among *sku* genes, *sku2* is strongly transcribed in liquid culture and throughout the various zones of the nodule, implying that *sku2* is expressed in free-living bacteria, in newly formed bacteroids and also in older and senescing bacteroids. In contrast, *sku3* and *sku4* seem to be preferentially expressed in the invasion zone.

IR irradiation induces foci formation of SKu2–YFP fusion protein in the free-living Rm1021

The particularly significant effect of a *sku2* deletion on IR survival and the high expression levels of the *sku2* gene (in both free-living cells and bacteroids) indicate that SKu2, probably acting together with LigD2, mediates the main NHEJ system in Rm1021. As many DNA repair and recombination proteins undergo subcellular localization to form foci after DNA damage (Smith *et al.*, 2001; Renzette *et al.*, 2005; Simmons *et al.*, 2007), we fused *yfpmut2* (encoding a monomeric derivative of YFP) to the genomic copy of *sku2*. The fusion gene was present in single copy at the *sku2* native locus in pSymB and expressed from the native *sku2* promoter.

To assess the response of the fusion protein to IR, we first re-examined the sensitivity of strains to lower doses of IR (≤ 200 Gy) (Fig. S1). Only 3% of the Rm1021 cells survived after treated

with 200 Gy of IR (Fig. 2). Under such severely damaging condition, subcellular localization of the fusion protein observed would mostly be occurring in dead cells. When cells were irradiated with 50 and 100 Gy of IR, significant populations (53% and 18% respectively) of the wild-type cells remained viable. We chose to use those doses of IR to treat the strain, in an effort to ensure that SKu2-YFP was examined under conditions where the localization pattern would be most relevant to surviving cells. Rm1021*sku2-yfpmut2* showed a wild-type level of survival, when cells were treated with 50 and 100 Gy of IR (Fig. S2). This observation indicates that the SKu2-YFP fusion protein retains the activity to mediate NHEJ *in vivo*.

As expected, stationary-phase cells showed stronger SKu2-YFP fluorescence than cells in logarithmic phase, confirming our observation with the *sku2*-promoter fusion (data not shown). A strain lacking SKu2-YFP exhibited a lower level of background fluorescence and did not have foci (data not shown). In the stationary-phase culture without IR treatment, the SKu2-YFP fluorescence was distributed throughout the cell. Interestingly, in ~4% ($n = 679$) of cells, one focus of SKu2-YFP was visible above the background fluorescence (Fig. 4A–C; Table 1). Strikingly, after exposure to 50 Gy of IR, SKu2-YFP foci formed in ~60% ($n = 726$) of cells (Fig. 4D–F). When the IR dose was increased to 100 Gy, an even larger population of cells (~75%, $n = 566$) contained SKu2-YFP foci (Table 1). A significant fraction (~17%, $n = 566$) of cells contained more than two foci per cell. Thus, formation of SKu2-YFP foci is IR dosage-dependent. To confirm the localization of DNA, cells were stained with the fluorescent DNA dye 4',6-diamidino-2-phenylindole (DAPI). As we expected, SKu2-YFP foci were 100% coincident with DNA (as represented in Fig. 4J–L). These observations suggested that SKu2 proteins accumulate on DSBs induced by IR irradiation *in vivo*. IR-inducible formation of SKu2-YFP foci was also observed in the Rm1021 $\Delta sku134$ background (data not shown). This observation suggests that SKu2 is recruited independently of other SKu proteins and likely forms a homo-dimer *in vivo*.

IR irradiation also induced formation of SKu2-YFP foci in log-phase cells, although these signals were faint compared with the foci intensity of cells in stationary phase (Fig. 4G–I). Interestingly, spontaneous formation (without IR treatment) of SKu2-YFP foci was observed in ~17% ($n = 474$) of cells during logarithmic phase, approximately fivefold more frequent than cells during stationary phase (Table 1). This could be caused by DSB formation as a result of replication stress in actively growing cells. Many studies have shown that replication fork collapse during DNA replication generates DSBs (Friedberg *et al.*, 2006).

To examine whether formation of SKu2-YFP foci was due to an increased abundance of SKu2-YFP protein, we performed immunoblot analysis to monitor cellular SKu2-YFP protein levels (Fig. 4M). SKu2-YFP protein levels in cells irradiated with IR (50 and 100 Gy) were indistinguishable from the level of SKu2-YFP in untreated cells. We therefore conclude that SKu2 foci are assembled through a redistribution of existing protein. This observation is also consistent with our result from the analysis of the *sku2* promoter activity (Fig. 3B), further indicating that expression of SKu2 is not regulated by DNA damage.

It has been reported that several proteins involved in HR localize to characteristic subcellular positions. For example, in *E. coli* and *B. subtilis*, RecA protein fused to GFP forms foci in response to DNA damage. RecA-GFP foci preferentially assemble at mid-cell, where DNA replication is expected to take place (Renzette *et al.*, 2005; Simmons *et al.*, 2007). To examine whether NHEJ apparatus localized to a specific subcellular location, we scored the position of SKu2-YFP foci related to the nearest cell pole in stationary-phase cells (Fig. 4N). Interestingly, more than 50% of SKu2-YFP foci were located near mid-cell, most likely because genomic DNA is more concentrated at mid-cell. Alternatively, the preferential location of SKu2-YFP foci could result from DSB hot spots at mid-cell or the collapse of a replication fork.

SKu2 can respond to IR irradiation in non-replicating bacteroids

To visualize the subcellular localization of the NHEJ apparatus during the chronic infection stage, alfalfa plants infected by Rm1021*sku2-yfpmut2* were irradiated with IR. Nodules were crushed immediately after IR treatment and directly observed with the epi-fluorescence/DIC microscope. Fully developed bacteroids with the characteristic morphology (enlarged and branched cells) were readily distinguishable from undifferentiated bacteria or plant debris (Fig. 5A and D). In the non-IR treated control, SKu2-YFP foci were observed in ~17% ($n = 915$) of bacteroids (Fig. 5A–C). Given that bacteroids contain up to 24 copies of genomes (Mergaert *et al.*, 2006), the frequency of spontaneous foci in bacteroids per genome is much less than that observed in free-living cells during logarithmic phase and probably less than in free-living stationary cells.

IR irradiation significantly induced formation of SKu2-YFP foci in a dose-dependent manner (Table 1). Particularly, populations of bacteroids containing multiple foci (≤ 3) increased sevenfold after exposed to 100 Gy of IR (Fig. 5D–F; Table 1). Although multiple nucleoids appeared to be randomly organized in bacteroid cells, SKu2-YFP foci were always coincident with DAPI staining (as represented in Fig. 5G–I). SKu2-YFP was therefore still able to form foci in response to the IR-induced DSBs in non-replicating bacteroids.

NHEJ is not required for Rm1021 to establish a symbiosis with alfalfa

The preferential distribution of NHEJ genes on pSymA and pSymB and their differential expression in alfalfa nodules suggest that they act during symbiosis as well as in the free-living stage. To test whether NHEJ plays a crucial role in symbiosis, alfalfa seedlings were inoculated with all *sku*- and *ligD*-defective mutants. No significant difference was observed in nodule number or in plant growth in comparison with the parental strain. Coinoculation experiments in which two strains are forced to compete for colonization of a single niche can often reveal defects not observed during single-strain inoculations. To examine the competitiveness of the NHEJ-deficient mutant related to the wild type, we coinoculated alfalfa seedling with a Rm1021 derivative expressing β -glucuronidase (Rm1021*bacA*::pJH104, a strain with pJH104 insertion in the *bacA* promoter region without disrupting *bacA* gene) and Rm1021 Δ *sku1234*, a strain lacking all *sku* genes, or Rm1021, the wild-type parent. By chromogenically staining nodules with X-gluc, we observed that Rm1021 Δ *sku1234* and Rm1021 competed with the *uidA*-tagged strain at a similar level, indicating that NHEJ is not required for nodulation competitiveness. The strong expression of *sku2* in nodule and the active foci assembly of SKu2-YFP in bacteroids suggest that Ku proteins contribute to maintain the genomic integrity of *S. meliloti* in planta, but are not required to establish a functional symbiosis with alfalfa under laboratory conditions.

Discussion

Bacterial NHEJ is a two-component DSB repair system that consists of a bacterial Ku protein and a multifunctional DNA repair enzyme LigD (or, to some extent, LigC) (Gong *et al.*, 2005). By tagging one of *S. meliloti* Ku proteins (SKu2) with YFP, we discovered that this component of NHEJ can undergo subcellular localization. Strikingly, SKu2-YFP fusion protein formed foci in response to IR irradiation (Fig. 4). The formation of SKu2-YFP foci increased in an IR dosage-dependent manner (Table 1). This observation, the first demonstration of DNA damage-dependent subcellular localization of NHEJ components in bacteria, clearly suggests that the Ku proteins accumulate at DSBs induced by IR. The significant intensity of foci above the background fluorescence suggests that foci are the result of increasing the local concentration of SKu2-YFP proteins at specific locations. Previous *in vitro* studies showed that multiple molecules of purified Ku (either bacterial or eukaryotic) could tightly bind to a single DNA substrate yielding multimeric Ku-DNA complexes (de

Vries *et al.*, 1989; Yaneva *et al.*, 1997; Weller *et al.*, 2002). This is likely due to the unique structure of a Ku dimer, an open ring-like molecule encircling the DNA that can freely slide along the DNA (Paillard and Strauss, 1991; Walker *et al.*, 2001). Recently, localization of eukaryotic Ku was visualized in hamster cells by using a physiologically functional eGFP–Ku80 fusion protein (Mari *et al.*, 2006). In agreement with our results, eGFP–Ku80, as a heterodimer with Ku70, accumulated at DSB induced by a focused laser beam. The FRAP (Fluorescence Recovery After Photobleaching; Houtsmuller and Vermeulen, 2001) assay revealed that accumulated eGFP–Ku80 could exchange with the soluble fraction, showing that Ku on DNA ends is in dynamic equilibrium with protein in solution.

Surprisingly, IR challenge induced SKu2-YFP foci during logarithmic phase (Fig. 4G–I), indicating that NHEJ also operates in replicating cells. Our experiments did not detect significant IR sensitization by *sku* deletions in logarithmically growing cultures (data not shown) as previously reported in *M. smegmatis* (Pitcher *et al.*, 2007c; Stephanou *et al.*, 2007). As DSBs are exclusively repaired through HR during logarithmic phase, HR might compensate for the loss of the NHEJ pathway under such conditions. Furthermore, we confirmed that Ku could still actively respond to DSBs in mature bacteroids, representing a non-replicating and terminally differentiated state (Fig. 5 and Table 1). Recently, it has been shown in *B. subtilis* that ongoing DNA replication was required to assemble RecA at a DSB and for functional HR (Simmons *et al.*, 2007). Our result presents the first indication that NHEJ is a DSB repair pathway functional in bacteria under conditions of chronic infection, where DNA replication is slow or absent. Although Rm1021 does not require NHEJ to establish symbiosis with alfalfa, it is yet possible that, in certain pathogen–host interactions, NHEJ allows repair of DSBs induced by the genotoxic defence of host cells, and may also be able to promote mutagenesis inside of host tissues, as DSB repair by bacterial NHEJ is highly error-prone (Gong *et al.*, 2005).

Rhizobia are unique among bacteria possessing NHEJ, as multiple Ku homologues are conserved in all genomes of rhizobia sequenced to date. Four homologues of Ku are detected in *Bradyrhizobium japonicum* USDA110, *Rhizobium etli* CFN42, *Rhizobium leguminosarum* bv. *viciae* 3841, *Mesorhizobium loti* MAFF303099 and *S. meliloti* Rm1021. *A. tumefaciens* C58, a plant pathogen closely related to *S. meliloti*, also possesses five Ku homologues. By deleting each *sku* gene, we showed that all four Ku proteins of Rm1021 were functional *in vivo* (Fig. 2A). Very recently, LigD and LigC proteins of *A. tumefaciens* C58 (AtuLigD1, D2, AtuLigC1, C2 and C3) were biochemically characterized (Zhu and Shuman, 2007). Purified *P. aeruginosa* Ku protein significantly enhanced the activity of AtuLigD2, C2 and C3 *in vitro*, indicating that those three proteins are capable of mediating NHEJ. Thus, those bacteria possess multiple sets of functional NHEJ components. It remains to be determined how each Ku protein interacts with various LigDs (or other Ku proteins) to mediate NHEJ.

Our identification of the involvement of multiple SKus in NHEJ in strain Rm1021 raised an important question: how are these *sku* genes regulated? Our analysis revealed that the transcriptional level of the *sku2* gene in free-living cells was significantly higher than that of *sku1* and the *sku3sku4* operon (Fig. 3A). Strong expression of *sku2* was also observed in all zones of alfalfa nodules, whereas *sku1* appeared silent in the nodule (Fig. 3C and D). Interestingly, expression of the *sku3sku4* operon was limited to the infection zone (Fig. 3E and F), suggesting that transcription of those genes is induced under a specific condition. Indeed, a microarray analysis of the response of *S. meliloti* to osmotic shock revealed that transcription of *sku3* was induced by 0.4 M of NaCl (Domínguez-Ferreras *et al.*, 2006). The induction of *ligD3* transcription was detected in nodules of *Medicago truncatula*, another host of Rm1021, by transcriptome analysis (Barnett *et al.*, 2004). By the histochemical staining of β -glucuronidase activity, we also observed that the expression of the *ligD3::uidA* fusion, which was not detected in alfalfa nodule (Fig. 3I), was induced in mature bacteroids in nodules elicited

on *M. truncatula* (data not shown). This suggests that some of NHEJ genes are regulated in a host-specific manner. It is interesting to note that MMC, a typical inducer of the SOS response, does not affect the expression of *sku* genes.

The physiological importance of NHEJ in the symbiotic system of *S. meliloti* Rm1021 and host plants remain unclear. Rm1021 $\Delta sku1234$, a strain lacking all *sku* genes, still can nodulate and establish a functional symbiosis with alfalfa under laboratory conditions. Nevertheless, by sealing DSBs in bacteroid DNA, NHEJ might be able to delay senescence and thus prolong nitrogen fixation capability, especially under stressful conditions. Given that mature bacteroids of Rm1021 cannot resume growth outside of the nodule (Mergaert *et al.*, 2006), it has been proposed that, upon the senescence of nodules, the undifferentiated population of bacteria in the nodule (invaded the tissue but remained not to be released into the host cytoplasm) are released into the environment to repopulate. As undifferentiated bacteria are thought to persist in lumens of the nodule tissue with little or no growth (Gage, 2004), it is possible that NHEJ is important for survival of those persisting bacteria as well. It is also possible that multiple NHEJ genes are evolved for fitness or survival under various stressful conditions found in the natural environment or for the symbiosis between *S. meliloti* and its alternative hosts. Expression patterns of *sku* genes suggest that each Ku protein of Rm1021 has a distinct role under different conditions. SKu2 probably mediates the main NHEJ pathway in Rm1021, given that SKu2 is highly expressed in both the free-living cells and bacteroids. This model is consistent with our observation that a deletion of *sku2* sensitized Rm1021 to IR at a higher extent than other *sku* single deletions (Fig. 2A). On the other hand, other NHEJ proteins could be auxiliary NHEJ components induced under specific environmental circumstances (such as under high salt stress or in nodules of *M. truncatula*). To this end, it would be interesting to examine the biochemical properties of each NHEJ component under stressful conditions.

Experimental procedures

Microbiological techniques

Strains and plasmids used in this study were listed in Table 2. *E. coli* recombinants were grown at 37°C on Luria–Bertani (LB) media/medium (Sambrook *et al.*, 1989). *S. meliloti* strain Rm1021 and its derivatives were raised at 30°C in/on M9 succinate medium with biotin or LBMC (LB supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂). Gentamycin (Gm), neomycin (Nm), streptomycin (Sm) and tetracycline (Tet) were added at concentrations of 50, 200, 500 and 15 µg ml⁻¹ respectively. Plasmids were mobilized into Rm1021 and derivative strains by tri-parental matings using pRK600 as the helper plasmid (Ditta *et al.*, 1980). Pfu turbo (Stratagene) was used in all PCR reaction followed by cloning. The amplified products were cloned into pCR-Blunt II-TOPO (Invitrogen) and verified by sequencing the inserts.

Construction of *sku* non-polar deletion mutants

To construct the deletion of *sku* genes, we amplified each full-length ORF with flanking regions (for *sku1* and *sku3*) or the 5' region and the 3' region with flanking regions separately (for *sku2* and *sku4*) by PCR using Rm1021 genomic DNA and the following primer pairs: $\Delta sku1$ (5'-GGATCCATGGCCCTGCGTCCCTATTGGAAAGGCT-3'/5'-GGATCCTCATTTGGTTCGCCCCGTTTCTCAGCCGCG-3'); $\Delta sku2$ -up (5'-TCTAGAAGAAATTCCATCAGGCGACGAGCG-3'/5'-CTGCAGATATAGCCCTTCCAACCTTGCCCTG-3'); $\Delta sku2$ -down (5'-GGATCCATGGCAGGCAAGTTGGAAGGGGCT-3'/5'-GGATCCAGGCTTTCTTCTTGGCCGCGGGTTAGC-3'); $\Delta sku3$ (5'-TTGGATCCCCTGGATATGCAGGGAGTTAGCCATGG-3'/5'-GCGGATCCAATTCTCGATGTCGATCGTCCTCGC-3'); $\Delta sku4$ -up (5'-AGTTCATTGACAGCGTCTCTGGCAAGCCG-3'/5'-

TTTCACCGCGCTCGTAACCTTTTCACCTCG-3'); $\Delta sku4$ -down (5'-TTGGATCCCCTGGATATGCAGGGAGTTAGCCATGG-3'/5'-CCGGATCCACTTCTACTTCCAGCCCGTCATGCAG-3'). Underlined nucleotides correspond to mismatches introduced to create selected restriction sites. To delete both *sku3* and *sku4* at the same time, the region containing *sku3* and *sku4* was also amplified with the primer set used for $\Delta sku3$ and $\Delta sku4$ -down. Most parts of *sku* coding sequences were deleted by restriction digestion of internal sites (for *sku1*, *sku3* and *sku3sku4*), or by combining two PCR fragments of 5' and 3' regions of genes (for *sku2* and *sku4*). DNA fragments containing in-frame *sku* deletions were subcloned into pJQ200-SK (Quandt and Hynes, 1993), and the resulting plasmids, pJQ- $\Delta sku1$, $\Delta sku2$, $\Delta sku3$, $\Delta sku4$ and $\Delta sku34$ were mobilized into Rm1021 or its derivatives by tri-parental mating. Transconjugants were first selected on LBMC plates containing Sm and Gm, then on LBMC plates containing Sm and 5% (w/v) of sucrose to select for double cross-overs. Replacement of wild-type *sku* genes with its deleted loci was confirmed by PCR. The position of deleted *sku* protein-coding regions on Rm1021 genome are shown in Fig. 1.

IR survival assay

Cultures of each strain were grown to saturation ($^{1cm}OD_{600}$ of > 1.5) in M9 succinate medium, washed in 0.85% NaCl, and diluted to $^{1cm}OD_{600}$ of 0.1 in 0.85% NaCl. Suspensions were aliquoted into 1.5 ml tubes and irradiated in a ^{60}Co gamma irradiator. Following IR irradiation of 0, 200, 400 and 600 Gy or 0, 10, 50, 100 and 200 Gy, cells were diluted in 0.85% NaCl, plated on LBMC, and incubated for 3 days before colony-forming units (cfu) were examined. Survival at each dose was determined as the percentage (%) of cfu formed by irradiated samples to cfu of the untreated sample. The results reported represent the means of at least three independent experiments.

Construction of reporter gene fusions

To construct promoter-*lacZ* fusions, predicted promoter regions of *sku1*, *sku2* and the *sku3sku4* putative operon were amplified by PCR using the following primer pairs: the *sku1* promoter (5'-TCTAGATCTGACACCGTCCGGAGATG GTC-3'/5'-CTGCAGGGCCATGGCGACCTCCGATCAA-3'); the *sku2* promoter (5'-TCTAGAAGAAATTCCATCAGGCGACGAGCG-3'/5'-CTGCAGATATAGCCCTTCCAACCTTGCCCTG-3'); the *sku3* promoter (5'-TCTAGACCTGGCAGGATGAGAAGGTCGTG-3'/5'-CTGCAGTGGCTAACTCCCTGCATATCCAG-3'); the *recA* promoter (5'-TCTAGATTCTGCGACTTCACCTGTGCTTGC-3'/5'-CTGCAGCGATTTGTCTCTACGAGCCGCAA-3'). To construct transcriptional-*uidA* fusions, internal regions of *sku1*, *sku2*, *sku3*, *sku4*, *ligD1P*, *ligD2*, *ligD3* and *ligD4* were amplified by PCR using the following primer pairs: *sku1* (5'-AGGATCCGGTGAAGGGCTATCAGCGA-3'/5'-TCGGGATCCAGTCCTTGGTCTGCTTT-3'); *sku2* (5'-CGGATCCACATGAAGCCCGTCGATC-3'/5'-TCGGCAAACCGGATCCCGAGATGCT-3'); *sku3* (5'-CCGGATCCCTTCCACACGGTCAAC-3'/5'-AGGGATCCCTCGAATTCTTCGGCCGAG-3'); *sku4* (5'-CGGATCCACACGCTCAACCGCAAGT-3'/5'-GGATCCATACGACGATGCCTTTGCC-3'); *ligD1P* (5'-GAGGATCCCGCCCGCTACCATGAAG-3'/5'-CTGGATCCAGTTGAGCGGCATCGAG-3'); *ligD2* (5'-ATGGATCCTGGCCCGTCTGAAGCGT-3'/5'-AAGGATCCAGCCGTTGAGATGCAGG-3'); *ligD3* (5'-CTGGATCCGACGACGGAATTTGAGG-3'/5'-

TCGGATCCAGGCAGGTGCAGTAGTC-3'); *ligD4* (5'-CAGGATCCGGTTCCTGGTTCAGAAGC-3'/5'-GTGGATCCGCCTTCCGAAAGCGCCA-3'). The cloned fragments were subcloned into the broad-host-range reporter vector pMP220 (for promoter-*lacZ* fusions) or the suicide *uidA* reporter vector pJH104 (for transcriptional-*uidA* fusions). Resulting plasmids were mobilized into Rm1021 or its derivatives by tri-parental mating. Transconjugants were selected on LBMC plates containing Sm and Tet (for pMP220 derivatives) or Sm and Nm (for pJH104). It must be noted that derivatives of pJH104 were inserted into native gene loci by single cross-over recombination, resulting in polar disruptions of corresponding genes. Insertions of pJH104 were confirmed by PCR. The position of the cloned *sku* promoters and pJH104 insertions in relation to their predicted protein-coding regions on Rm1021 are shown in Fig. 1. To replace the genomic copy of *sku2* with *lacZ*, the XbaI-SacI fragment of pMP-*sku2p* containing the *sku2* promoter-*lacZ* fusion was ligated with the XbaI-SacI fragment of pJQ- Δ *sku2*. The resulting plasmid was integrated into Rm1021 by double cross-over as described above, to give rise of Rm1021*sku2* Δ *lacZ*.

β -Galactosidase assay in free-living Rm1021 strains

The β -galactosidase activity of Rm1021 transconjugants was assayed according to Miller (1972). Overnight exponentially growing cultures ($^{1\text{cm}}\text{OD}_{600}$ of 0.4–0.6) were initially adjusted to $^{1\text{cm}}\text{OD}_{600}$ of 0.1 in LBMC (0 h after subculture). β -Galactosidase activities were monitored 1, 3, 6, 9 and 24 h after subculture. To induce SOS response, $0.2 \mu\text{g ml}^{-1}$ of MMC was added 1 h after subculture.

β -Glucuronidase assay in nodules

Seedlings of alfalfa (*Medicago sativa* cv. Iroquois) were inoculated with Rm1021 derivatives on Petri dishes containing Jensen agar as described previously (Leigh *et al.*, 1985; Pellock *et al.*, 2000). Plants inoculated with Rm1021 derivatives containing pJH104 insertions showed a wild-type phenotype for nodulation. Four-week-old nodules were excised, sectioned and incubated in X-gluc staining buffer [1 mM 5-bromo-4-chloro-3-indoyl- β -d-glucuronide, 0.1 M Na-phosphate buffer (pH 7), 10 mM EDTA, 0.5 mM K-Ferricyanide, 0.5 mM K-Ferrocyanide, 0.1% Triton X-100] at 37°C for overnight.

Construction of Rm1021 *sku2-yfpmut2*

The 3' region of *sku2* coding sequence and the region downstream of *sku2* (starting from the stop codon of *sku2*) were amplified by PCR using the following primer pairs: *sku2-yfpmut2*-up (5'-GGGCCCCATCAGACTGAGTCTCGTGAGCTGTCC-3'/5'-CTCGAGGGCTTTCTTCTTGGCCGCGGGTTTAGCAG-3'); *sku2-yfpmut2*-down (5'-CTGCAGATGGCGCTTGCCGGTCGGACATTCGGTATC-3'/5'-TCTAGATAACGCATCCACCAGCGGCGGGCTTCGGC-3'). Two fragments were subcloned in pJQ200SK and combined with the XhoI-PstI fragment containing *yfpmut2*, encoding the A206K missense mutation generating monomeric YFP, of pKL183 (Lemon and Grossman, 2000). The resulting pJQ200SK derivative carried *sku2-yfpmut2* followed by the downstream sequence of native *sku2* locus. Replacement of wild-type *sku2* locus with *sku2-yfpmut2* was achieved by a similar procedure as in construction of *sku* deletion strains.

Live cell microscopy

Aliquots of cells grown in M9 medium supplemented with succinate were stained with the vital membrane dye FM4-64 (Molecular Probes) and the DNA stain DAPI. The following Chroma filter sets were used: 41029 for YFP, 41002C for FM4-64 and 31000 for DAPI. Exposure time for SKu2-YFP fusion protein was 3 s. Images were acquired, colourized and merged using OpenLab software (Improvision). To assess the effect of IR irradiation in free-

living cells, aliquots of M9 cultures of stationary phase ($^{1\text{cm}}\text{OD}_{600}$ of > 1.5) or log-phase ($^{1\text{cm}}\text{OD}_{600}$ of $0.4\text{--}0.5$) were irradiated with IR of 0, 50 and 100 Gy and observed under the microscope.

For bacteroids, whole alfalfa plants on Petri dishes containing Jensen agar were irradiated in a ^{60}Co gamma irradiator. Nodules were immediately harvested, crushed, and bacteroids were observed under the microscope. Positions of foci were scored after colorization and merging of microscopic images. All data presented here are cumulative from at least two independent experiments, each of which gave nearly identical results.

Immunoblot analysis of SKu2-YFP protein

Rm1021*sku2-yfpmut2* and Rm1021 were grown in M9 medium supplemented with succinate. Cells of the stationary phase were irradiated by IR of 50 and 100 Gy, then lysed by boiling in the presence of 1% SDS. Total proteins were acetone precipitated and quantified by Bradford assay (Bio-Rad). One milligram of protein from each extract was separated on SDS-polyacrylamide (4% to 20% w/v) gels. For immunodetection, separated proteins were transferred on PVDF Immobilon-P membranes (Millipore Corporation) and probed with 1/4000 working dilutions of the anti-GFP (Abcam). Horseradish peroxidase-labelled goat anti-rabbit immunoglobulin antibodies of the ECL kit (Amersham Pharmacia Biotech) were used as secondary antibodies. Reactions were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Genetic organization of loci encoding proteins of NHEJ apparatus. The genome of *S. meliloti* Rm1021 consists of three replicons: one chromosome, two megaplastids (pSymA and pSymB) (Galibert *et al.*, 2001). A blast database search detected four ORFs encoding close homologues of bacterial Ku in megaplastids: SMA0426 (*sku1*) in pSymA; SMb20686, SMb21406 and SMb21407 (*sku2*, *sku3* and *sku4* respectively) in pSymB. Six ORFs encoding LigD homologues were also identified: SMA0414, SMA0417 and SMA0424 (*ligD1L*, *ligD1P* and *ligC*) in pSymA; SMb20685 and SMb21044 (*ligD2* and *ligD3*) in pSymB; and SMC03959 (*ligD4*) in the chromosome. Genes are represented by arrows showing the direction of transcription and coloured depending on the predicted function of their encoding proteins: purple, bacterial Ku protein; green, DNA polymerase domain; yellow, ATP-dependent DNA ligase domain; blue, DNA exonuclease domain. The regions deleted in *sku* deletion mutants are marked with red lines. Regions subcloned in pMP220 are marked with blue lines. Arrowheads represent locations where pJH104 derivatives were inserted.

Fig. 2.

IR sensitivity of *S. meliloti* Ku (*sku1*, *sku2*, *sku3* and *sku4*) deletion strains.

A. Stationary-phase cells of *S. meliloti* wild type (Rm1021), *sku1*, *sku2*, *sku3*, *sku4* deletion mutant (Rm1021 $\Delta sku1$, Rm1021 $\Delta sku2$, Rm1021 $\Delta sku3$ and Rm1021 $\Delta sku4$) were irradiated with γ-rays. The percentage survival for each strain over the indicated range of doses was determined, and the mean from at least three independent experiments was plotted. Error bars denote standard error from the mean for each point.

B. IR sensitivity of the *sku1sku3* double mutant (Rm1021 $\Delta sku13$), *sku1sku3sku4* triple mutant (Rm1021 $\Delta sku134$) and *sku1sku2sku3sku4* quadruple mutant (Rm1021 $\Delta sku1234$). The wild type (Rm1021) and Rm1021 $\Delta sku2$ were also treated as internal controls.

Fig. 3.

Expression of *S. meliloti* NHEJ genes in free-living cells and bacteroids. Panels A and B represent the levels of β-galactosidase activity (given in 10³ Miller's units) for the promoter region of *sku1*, *sku2*, *sku3* and *recA* fused to *lacZ* respectively. Assays were performed at 1, 3, 6, 9 and 24 h after subculture. In B, 0.2 μg ml⁻¹ of MMC was added to induce SOS response. The values reported represent the means of three independent experiments with standard errors (error bars). Growth curves of a representative strain (monitored by 1cm³ OD₆₀₀) were also shown (red curves). Panels C–J represent histochemical localization of β-glucuronidase activity in nodule hand-sections. Nodules were harvested from alfalfa plants infected with Rm1021 strains carrying NHEJ gene-*uidA* fusions (Rm1021 *sku1*::pJH104,

Rm1021*sku2*::pJH104, Rm1021*sku3*::pJH104, Rm1021*sku4*::pJH104, Rm1021*ligD1P*::pJH104, Rm1021*ligD2*::pJH104, Rm1021*ligD3*::pJH104 and Rm1021*ligD4*::pJH104; C–J respectively). β -Glucuronidase activity was visualized as blue precipitates of the chromogenic substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). A total of 30–50 nodules from five plants were examined for each fusion. The meristematic zone of nodule is marked with white asterisks. Scale bar represents 0.5 mm.

Fig. 4. Subcellular localization of SKu2 in free-living *S. meliloti* cells. Panels A–L represent subcellular localization of SKu2–YFP fusion proteins in free-living *S. meliloti* by epi-fluorescence microscopy. Rm1021.*sku2-yfpmut2* was grown in M9 medium supplemented with succinate. Cells of the stationary phase (A–E and J–L) or the logarithmic phase (G–I) were irradiated by 50 Gy of IR (D–I), 100 Gy of IR (J–L) or mock-treated (A–C). Localization of SKu2–YFP was visualized in living cells as represented in green (B, E and H) or white (K). The cell membrane was stained by FM4-64 as represented in red (A, D and G). Cellular DNA was stained by DAPI as represented in blue (J). Epi-fluorescence views of different filters were merged to analyse relative localization (C, F, I and L). No focus was observed in cells of the

Rm1021 wild-type strain (data not shown). Scale bar represents 2 μ m. Panel M represents immunoblot analysis of SKu2-YFP protein levels in Rm1021 *sku2-yfpmut2* after treatment with the indicated level of IR. Cells of stationary phase were treated with IR of 0, 50 and 100 Gy. Whole-cell extracts were blotted onto PVDF membrane and probed with anti-GFP (Abcam). One milligram of total protein from each cell extracts was loaded. Although few signals of endogenous proteins cross-reacting with anti-GFP were detected commonly in extracts from both Rm1021 *sku2-yfpmut2* (*sku2-yfpmut2*) and the wild-type control (Rm1021), strong signals of SKu2-YFP, a protein of expected molecular weight of 60 kDa, were only detected in extracts of Rm1021 *sku2-yfpmut2* (indicated with a open arrow head). Panel N represents the subcellular position of SKu2-YFP as a percentage of cell length for single-focus cells ($n = 95$). Cells of stationary phase were treated with IR of 50 Gy. The relative position of SKu2-YFP foci in each cell was determined as a ratio (%) of the distance between foci and the proximal pole to the longitudinal length of the cell.

Fig. 5.

Subcellular localization of SKu2 in bacteroids. Panels A–I represent subcellular localization of SKu2–YFP fusion proteins in *S. meliloti* bacteroids by epi-fluorescence/DIC microscopy. Alfalfa plants infected with Rm1021*sku2-yfpmut2* were irradiated by 100 Gy of IR (D–F), 50 Gy of IR (G–I) or mock-treated (A–C). Nodules were immediately harvested and crushed. Long, blanched bacteroid cells were visually distinguished from non-differentiated *S. meliloti* cells or plant-derived materials (A and D). In contrast to the free-living cells, the membrane of bacteroids could not be stained by FM4-64 (data not shown). Localization of SKu2–YFP was visualized in bacteroids as represented in green (B and E) or white (H). Cellular DNA was stained by DAPI as represented in blue (G). Epi-fluorescence views of different

filters and DIC views were merged to analyse relative localization (C, F and I). No focus was observed in bacteroid cells of the Rm1021 wild-type strain (data not shown). Scale bar represents 2 μm .

Table 1

The population of cells with SKu2-YFP foci increase in an IR dose-dependent manner.

Ionizing radiation (Gy)Growth phase	No. of cells	Percentage of cells with <i>n</i> foci			
		0	1	2	≥ 3
0Stationary	679	96	4	< 1	ND
50	726	41	49	9	< 1
100	566	25	58	17	< 1
0Logarithmic	474	74	23	3	< 1
50	647	52	37	8	3
100	550	27	49	20	4
0Bacteroid	915	83	9	5	3
50	919	59	21	11	9
100	970	40	23	17	20

Rm1021*sku2-yfpmur2* was grown in M9 medium supplemented with succinate. Stationary-phase or the logarithmic phase cells were irradiated by 0, 50 and 100 Gy of IR. For bacteroids, alfalfa plants infected with Rm1021*sku2-yfpmur2* were irradiated, and nodules were immediately crushed to observe bacteroids.

Table 2

Strains and plasmids used in this study.

	Relevant characteristics	Reference
<i>Sinorhizobium meliloti</i> strains		
Rm1021	Sm ^R derivative of SU47	Meade <i>et al.</i> (1982)
Rm1021Δ <i>sku1</i>	Deletion mutant of Rm1021 where the 0.3 kb <i>Sall</i> - <i>XhoI</i> fragment was removed from <i>sku1</i>	This study
Rm1021Δ <i>sku2</i>	Deletion mutant of Rm1021 where the 0.5 kb fragment was removed from <i>sku2</i>	This study
Rm1021Δ <i>sku3</i>	Deletion mutant of Rm1021 where the 0.5 kb <i>XhoI</i> fragment was removed from <i>sku3</i>	This study
Rm1021Δ <i>sku4</i>	Deletion mutant of Rm1021 where the 0.6 kb <i>Sall</i> - <i>XhoI</i> fragment was removed from <i>sku4</i>	This study
Rm1021Δ <i>sku13</i>	Rm1021Δ <i>sku1</i> strain whose <i>sku3</i> was also deleted as in Rm1021Δ <i>sku3</i>	This study
Rm1021Δ <i>sku134</i>	Rm1021Δ <i>sku1</i> strain where the 1445 bp <i>XhoI</i> fragment was removed from <i>sku3</i> - <i>sku4</i> region	This study
Rm1021Δ <i>sku1234</i>	Rm1021Δ <i>sku134</i> whose <i>sku2</i> was also deleted as in Rm1021Δ <i>sku2</i>	This study
Rm1021 <i>sku2</i> Δ <i>lacZ</i>	Derivative of Rm1021 whose <i>sku2</i> and 0.6 kb of the downstream ORF was replaced with <i>lacZ</i>	This study
Rm1021 <i>sku1</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>sku1</i> at 628 bp downstream from the start codon	This study
Rm1021 <i>sku2</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>sku2</i> at 561 bp downstream from the start codon	This study
Rm1021 <i>sku3</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>sku3</i> at 649 bp downstream from the start codon	This study
Rm1021 <i>sku4</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>sku4</i> at 478 bp downstream from the start codon	This study
Rm1021 <i>ligD1P</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>ligD1P</i> at 703 bp downstream from the start codon	This study
Rm1021 <i>ligD2</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>ligD2</i> at 1003 bp downstream from the start codon	This study
Rm1021 <i>ligD3</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>ligD3</i> at 1099 bp downstream from the start codon	This study
Rm1021 <i>ligD4</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in <i>ligD4</i> at 962 bp downstream from the start codon	This study
Rm1021 <i>sku2-yfpmut2</i>	Rm1021 derivative where <i>sku2-yfpmut2</i> replaced the native <i>sku2</i>	This study
Rm1021 <i>bacA</i> ::pJH104	Derivative of Rm1021 with a pJH104 insertion in the <i>bacA</i> promoter region without disrupting <i>bacA</i>	G.P. Ferguson (unpublished)
Plasmids		
pJH104	Suicide vector containing a promoterless <i>uidA</i> , Km/Nm ^R	Ferguson <i>et al.</i> (2005)
pMP220	IncP expression vector containing a promoterless <i>lacZ</i> gene, Tc ^R	Spaink <i>et al.</i> (1987)
pMP- <i>sku1</i> p	The <i>sku1</i> promoter region cloned in pMP220 as a 0.5 kb <i>XbaI</i> - <i>PstI</i> fragment	This study
pMP- <i>sku2</i> p	The <i>sku2</i> promoter region cloned in pMP220 as a 0.5 kb <i>XbaI</i> - <i>PstI</i> fragment	This study
pMP- <i>sku3</i> p	The <i>sku3</i> promoter region cloned in pMP220 as a 0.5 kb <i>XbaI</i> - <i>PstI</i> fragment	This study
pMP- <i>recA</i> p	The <i>recA</i> promoter region cloned in pMP220 as a 0.5 kb <i>XbaI</i> - <i>PstI</i> fragment	This study