Supporting Online Material for G. Weller et al., 1074584

Materials and Methods

Construction of YkoU and YkoV mutant B. subtilis strains. Strains BFS1845 and BFS1846 were constructed using the plasmid pMUTIN4 (S1). PCR-amplified fragments of an internal region of YkoU or YkoV were cloned into the Eco RI–Bam HI sites of pMUTIN4. A 32–base pair (bp) PCR fragment of the YkoU gene, synthesized using the primers SM44 (5'-CCGGAATTCTCAATGGACTGGACC-3') and SM45 (5'-CGCGGAGATGGACATATCTCCTCAGAGAC-3'), was cloned into pMUTIN4 to make the plasmid pDU1845. A 253-bp fragment of the YkoV gene, synthesized with the primers SM46 (5'-CCGGAATTCTTCAAGGAAATGGATCAGG-3') and SM47 (5'-CGCGGAGATTGAGATCCAGGCTACACC-3'), was cloned into pMUTIN4 to make pDU1846. The plasmids, pDU1845 and pDU1846, were propagated in E. coli and were then transformed into the B. subtilis chromosome generating strains BFS1845 and BFS1846. Strains were verified by PCR. In these strains the gene of interest is inactivated and a transcriptional lacZ fusion has been generated in each case. B. subtilis strain SL7360 [recA::neo; (S2)] was generously provided by P. J. Piggot (Temple University School of Medicine, Philadelphia). The parent strain of SL7360 is B. subtilis BR151 (trpC2 lys-3 metB10). In order to make all the strains isogenic, chromosomal DNA from SL7360 was transformed into B. subtilis 168 to generate SL7360-168. Chromosomal DNA from SL7360 was also transformed into BFS1845 and BFS1846 to create the strains ES33 and ES34, respectively. Sequence analysis indicates that YkoV and YkoU are probably in the same operon. It should be noted that integration of the pMUTIN4 plasmid into YkoV generated a strain (ykoV) in which YkoV is inactivated and the downstream gene, YkoU, is under the control of the IPTG-inducible P_spac promoter. When this strain is grown in the absence of IPTG neither YkoV or YkoU are expressed and this represents the “double-mutant” (ykoV ykoU). When the strain is grown in the presence of IPTG ykoU is expressed under the control of the P_spac promoter. This is also the case for the recA ykoV strain.

Ionizing radiation assays. Stationary phase cultures of B. subtilis wild type, ykoV ykoU or ykoV ykoU double mutants, and B. subtilis recA mutant cells were treated with the indicated doses of x-rays using a Faxitron (3.15 Gy/min at 120 kVp), which uses beryllium as its source of radiation. The irradiated cells (50 µl of 1:15000 dilution) were plated using a spiral plater (WASP) and grown overnight at 37°C. The percentage survival, post radiation, for each strain was determined by counting the number of colony-forming units.

Cloning of Rv0937c and Rv0938 ORFs. Full-length sequences for M. tuberculosis Rv0937c and Rv0938 were amplified by PCR from H37Rv genomic DNA using the following primers: Rv0937c (M. tuberculosis Ku, 274 amino acids, 30.9 kD) was amplified using 5' primer (5'-CAT ATG CGA GCC ATT TGG ACG GG-3') and 3' primer (5'-GGA TCC TCA CGG AGG CTG TGG GAC G-3'). Rv0938 (M. tuberculosis ligase, 759 amino acids, 83.6 kD) was amplified using 5' primer (5'-CAT ATG GGT TCG GCC TCG GAG GAC CA-3') and 3' primer (5'-GGA TCC TCA TCC GCG CAC CAC CTC ACT GG-3'). Note that the 5' primers contained an Nde I site, and the 3' primers contained a Bam HI site. PCR products were cloned into pET16b (Novagen). All DNAs cloned from PCR products were sequenced to confirm that no mutations were introduced during PCR. Proteins overexpressed from this vector carry an extra 21 amino
acids (2.5 kD) at the NH₂-terminus of the protein, due to addition of a 10-His tag and a Factor Xa cleavage site.

Overexpression of RV0937c and RV0938. Recombinant protein was produced by first transforming *E. coli* B834 (DE3) pLysS cells (Novagen) with the pET16b plasmid (containing either Rv0938 or Rv0937c) and then selecting a single colony which was grown overnight at 37°C in 5 ml LB broth supplemented with ampicillin at 100 µg/ml and chloramphenicol at 34 µg/ml. The overnight culture was used to inoculate 1 liter of LB broth supplemented with ampicillin and chloramphenicol as before. This culture was grown at 37°C until an OD₆₀₀ of 0.6 was achieved. At this point the culture was removed from the incubator and cooled to room temperature in a water bath and IPTG was added to a final concentration of 0.5 µM, to induce the production of the recombinant protein. The culture was then returned to the incubator and grown overnight at 28°C. The cells were pelleted for 20 min at 4000g.

Purification of Mt-Ku (RV0937c). After sonication, the cell supernatant was treated with 60% of a saturated ammonium sulfate solution, incubating on ice for 1 hour. This was spun down, and the pellet was carefully resuspended in buffer A (50 mM Tris pH 7.5, 60 mM NaCl, 30 mM imidazole, 17 µg/ml PMSF, 34 µg/ml benzamidine). The resuspended was then loaded onto a nickel agarose (Qiagen) column, washed with 60 mM imidazole, and the protein eluted with 300 mM imidazole. The 300-mM peak was then loaded onto a DEAE Sepharose fast flow column. The Ku protein eluted between 200 and 300 mM NaCl.

Purification of Mt-Lig (RV0938). After sonication, the cell debris was removed by centrifugation. The supernatant pellet was then loaded onto a nickel agarose column (Qiagen), washed with 60 mM imidazole, and the protein eluted with 300 mM imidazole. The 300 mM peak was then loaded onto a 5 ml Hi-Trap Q-Sepharose column (Amersham Biosciences). The ligase eluted at around 300 mM NaCl, which corresponded to a single protein band at approximately 83 kD, the predicted size for the full length Rv0938 gene product.

Double-stranded ligation assay. Equimolar concentrations of Mt-Lig, Ligase IV/XRCC4 or T4 DNA ligase were incubated for 2 hours in 30 µl reaction mixture (50 mM Triethanolamine, pH 7.5, 2 mM Mg(OAc)₂, 2 mM DTT, 0.1 mg/ml BSA) or 1× reaction buffer for T4 DNA ligase (Roche) with 70 fmol of DNA ([γ⁻³²P]ATP labeled on the 5’ end). Double-stranded DNA fragments were produced from the Bluescript plasmid (Stratagene) to give substrates of 53 bp, and 445 bp, and 2.56 kbp with 4 bp overhangs at each end, and a 157-bp substrate with a 4-bp and a 2-bp overhang. These cohesive ends were not complementary to limit circularization. Bluescript was digested initially with the restriction enzymes Pst I and Afl III (NEB) to produce the 445-bp and 2.56-kbp DNA fragments. The large fragment produced by the first digestion was subjected to a second double digest with Kpn I and Pvu II (NEB) to produce 53 bp and 157 bp fragments. After incubation, the reactions were deproteinized, phenol/chloroform extracted and precipitated with Pellet-Paint co-precipitant (Novagen). Aliquots of the reactions were run on 0.8% agarose gels. Dried gels were analyzed and quantified using a STORM PhosphorImager (Molecular Dynamics). Reactions with Ku heterodimer were preincubated for 15 min on ice with indicated amounts of Ku heterodimer, and ligation reaction was started by adding the enzyme and transfer to 37°C.
**Plasmid repair assay.** pUC18 plasmid DNA was cut with Eco RI and gel purified. The cut plasmid was then incubated with Rv0937c, Rv0938, or both proteins in 1× standard T4 ligase buffer. Uncut plasmid, cut plasmid, and cut plasmid re-ligated with T4 DNA ligase were used as controls. The reactions were incubated at room temperature for one hour, and the reaction stopped by heating at 65°C for 20 min. From each reaction mixture, 5 µl was transformed into XL1 Blue *E. coli* and incubated at 37°C for 1 hour, before being plated onto ampicillin/tetracycline agar plates and grown at 37°C overnight. The number of colony-forming units was then determined.

**Supporting Online Text**

Based on recent sequence alignment studies, it is now evident that the bacterial Ku proteins represent a highly conserved domain at the centre of the larger eukaryotic Ku proteins (S3, S4). In order to understand more about the structure and function of this domain, we mapped the location of bacterial Ku sequences onto the crystal structure of the Ku heterodimer (S5; Fig. S3) based on the alignments of the bacterial and human Ku sequences (S4). The Ku core domain lacks the NH2-terminal VWA domain and extreme COOH-terminal domains (SAP and DNA-pk) but, importantly, retains all the structural elements required for dimerization and DNA-binding (Fig. S3). Consistent with this “model”, our biochemical data shows that Mt-Ku exists as a homodimer that binds specifically to dsDNA ends. It has also been reported that the equivalent truncated versions of eukaryotic Ku, consisting only of the central β-barrel domain and COOH-terminal region, retain both heterodimerization and DNA end-binding activities (S6–S9). Interestingly, several bacteria, including *Mesorhizobium loti, Sinorhizobium loti* and *Agrobacterium tumefaciens*, contain two distinct bacterial Ku genes in the same operon (S10), suggesting the possible existence of heterodimeric Ku complexes in these species.
**Supplemental Fig. 1.** Purification of Mt-Ku and Mt-ligase. Molecular Weight Markers (MW; 17, 30, 42, 66 and 77 kD, respectively), Lanes 1-4 (Mt-Ku purification): induced whole cell supernatant, ammonium sulfate precipitated pellet, nickel-agarose elution peak fraction and DEAE-Sepharose elution peak fraction. Lanes 5-7 (Mt-ligase purification): induced whole cell supernatant, nickel-agarose elution peak fraction and Q-Sepharose fraction.
Supplemental Fig. 2. Mt-Ku exists as a homodimer in solution. The elution volume for Mtb Ku, used to calculate the $K_{AV}$ value. The $K_{AV}$ values of a range of standard protein were calculated using the equation $(V_e - V_o)/(V_t - V_o)$ where $V_e$ is the elution volume of the protein, $V_o$ is the void volume of the column, as calculated using Blue Dextran 2000, and $V_t$ is the total bed volume of the column. $K_{AV}$ was plotted versus Log of molecular mass (daltons) to create a standard curve, onto which the $K_{AV}$ value of Mt-Ku could be plotted. Plotting this curve revealed that Mt-Ku and Mt-Lig eluted with apparent molecular weights of ~61 kD and ~82 kD, respectively, which suggests that Ku exists as a homodimer complex and Mt-Lig as monomer. ● = Mt-Ku, ▲ = Mt-Lig
Supplemental Fig. 3. Structure of the bacterial Ku homodimer-DNA complex. A structure-based sequence alignment was used to model the structure of the Mt-Ku homodimer. The smaller bacterial Ku homologs do not possess the extreme NH$_2$-terminal von Willebrand domains (VWA) or the COOH-terminal SAP (Ku70) and DNA-PKcs binding (Ku80) domains present in the larger eukaryotic Ku proteins. These domains have been removed from the structure of the human Ku complex (S5; PDB ID: 1jey) to leave a truncated Ku complex (consisting of truncated Ku70: residues 262-521 and Ku80: residues 254-515), which we believe represents a close approximation of the bacterial Ku homodimer. Importantly, the structure of the proposed bacterial Ku complex retains the structural elements required for both dimerization and DNA binding.
Supplemental Fig. 4. Mt-Ku binds dsDNA. Electrophoretic-mobility shift assay (EMSA) was employed to characterize the ability of Mt-Ku complex to bind DNA. Reactions contained 90 fMol $^{32}$P radiolabeled ds 33-mer (3′ or 5′ overhang) plus increasing amounts of Mt-Ku: (0.02, 0.2, 2, or 8 pMol for 3′-DNA shifts and 0.02, 0.2, 2, 4 or 8 pMol with 5′-DNA shifts). Human Ku70/80 complex (hKu) can also shift the 33-mer. Reactions contained 90 fMol ds labeled 33-mer and increasing amounts of hKu (0, 1, 10, 50, 100 fMol). Reactions were carried in a buffer consisting of 25 mM Tris pH 7.5, 100 mM NaCl, 30 mM KCl, 0.1 mM EDTA, 0.05% Triton X-100 500 µg/ml BSA, 2% glycerol, and 2mM DTT.
**Supplemental Fig. 5.** Ku-associated ligases join DSBs in an ATP-dependent reaction. A sequence alignment of the adenylation domain of the eukaryotic ligase IV from *Saccharomyces cerevisiae* with some of the Ku-associated bacterial ligases (AF, *Archaeglobus fulgidus*; BS, *Bacillus subtilis*; BH, *Bacillus halodurans*; mll, *Mesorhizobium loti*; RV, *Mycobacterium tuberculosis* and PA, *Pseudomonas aeruginosa*). The five sequence elements, designated motifs I, III, IIIa, IV, V, conserved in DNA ligases are shown. The position of the conserved active site lysine is highlighted by the box. Asterisk (*) indicates identical or conserved residues in all sequences in the alignment; colon (:) indicates conserved substitutions; and period (.) indicates semiconserved substitutions.

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Supplemental Fig. 6. Mt Ku stimulates ligation in an in vitro plasmid repair assay. Eco RI–cut plasmid pUC 18 was incubated with Mt-Ku, Mt-Lig, or both proteins. Controls used: uncut plasmid, cut plasmid without any protein, and cut plasmid with T4 DNA ligase. The reactions were incubated at 25°C for 1 hour, and the reaction stopped by heating at 65°C for 15 min. Equimolar amounts of each reaction were transformed into E. coli (XL1 blue), plated onto ampicillin/tetracycline agar plates and grown at 37°C overnight. The plates were scanned, and the number of colonies was counted.
References


