Distinct DNA repair pathways involving RecA and nonhomologous end joining in *Mycobacterium smegmatis*

Malgorzata Korycka-Machala[1], Anna Brzostek[1], Sylwia Rozalska[2], Anna Rumijowska-Galewicz[1], Renata Dziedzic[1], Richard Bowater[3] & Jaroslaw Dziadek[1]

[1]Centre of Medical Biology, Polish Academy of Sciences, Lodowa, Poland; [2]Department of Industrial Microbiology & Biotechnology, University of Lodz, Banacha, Poland; and [3]School of Biological Sciences, University of East Anglia, Norwich, UK

Correspondence: Jaroslaw Dziadek, Centre of Medical Biology, Polish Academy of Sciences, 93-232 Lodz, Lodowa 106, Poland. Tel.: +48 42 2723610; fax: +48 42 2723630; e-mail: jdziadek@cbm.pan.pl

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Abstract

Mycobacterium smegmatis was used to study the relationship between DNA repair processes involving RecA and nonhomologous end joining (NHEJ). The effect of gene deletions in *recA* and/or in two genes involved in NHEJ (*ku* and *ligD*) was tested on the ability of bacteria to join breaks in plasmids transformed into them and in their response to chemicals that damage DNA. The results provide *in vivo* evidence that only NHEJ is required for the repair of noncompatible DNA ends. By contrast, the response of mycobacteria to mitomycin C preferentially involved a RecA-dependent pathway.

Introduction

Double-strand breaks (DSBs) are a specific type of DNA damage that may form during problems encountered during DNA metabolism (e.g. when replication forks meet obstacles or single-strand breaks) or due to the action of exogenous chemicals or radiation (Jackson, 2002; Lieber *et al.*, 2003). DSBs sometimes arise during normal physiological processes, for example in V(D)J recombination of immunoglobulin genes, but they are also particularly potent in causing problems for cellular metabolism. All organisms have a range of cellular pathways that repair DSBs. The majority of repair of DSBs is performed by two pathways, homologous recombination (HR) and nonhomologous end joining (NHEJ).

In prokaryotes, it has been assumed that the main pathway that promotes repair of DSBs is HR, which uses RecA to facilitate the exchange of single strands of DNA. However, RecA has other distinct biochemical activities that are used in other cellular pathways in bacteria, most notably the SOS response (Lusetti & Cox, 2002). Recent analysis of genome sequences identified potential prokaryotic homologues of two proteins known to be involved in NHEJ, namely Ku and an 'NHEJ-type' of DNA ligase (Aravind & Koonin, 2001; Doherty *et al.*, 2001; Weller & Doherty, 2001; Bowater & Doherty, 2005; Hefferin & Tomkinson, 2005). Mycobacteria are the organisms in which prokaryotic NHEJ has been described best. Biochemical analyses of recombinant versions of the proteins from *Mycobacterium tuberculosis* suggest that MtKu and MtLigD are able to form a functional NHEJ-processing system (Weller *et al.*, 2002; Della *et al.*, 2004). In support of this, deletion of *ligD* in both *M. tuberculosis* and *Mycobacterium smegmatis* generated cells that were defective in NHEJ (Gong *et al.*, 2004, 2005). Use of the *M. smegmatis* system showed that NHEJ of blunt-end and complementary 5′-overhang DSBs involves several DNA end-remodelling activities and is highly mutagenic (Gong *et al.*, 2005).

It is clear that many genes are involved in the response to DNA damaging agents, as demonstrated for the effect of mitomycin C (MMC) on *Escherichia coli* (Khil & Camerini-Otero, 2002). Analysis of the effects of MMC on different mycobacteria has identified varying effects, with not all being related to expression of *recA* (Papavinasasundaram *et al.*, 2001; Davis *et al.*, 2002; Rand *et al.*, 2003). Moreover, studies in yeast and mammalian cells identified the involvement of different pathways in the repair of interstrand crosslinks (ICLs), with NHEJ being important during some

parts of the cell cycle (De Silva *et al*., 2000; McHugh *et al*., 2000). The potential for interactions between different DNA repair pathways leads to questions about the extent of overlap and cross-talk between them. In this study, we use *M. smegmatis* to examine the relationship between pathways involving RecA or NHEJ, which are known to be able to repair similar types of damage.

Materials and methods

Bacterial strains and growth conditions

All strains used in this study were based on *Mycobacterium smegmatis* mc^2155 (Snapper *et al*., 1990) and were cultured in Middlebrook 7H9 broth supplemented with albumin-dextrose-sodium chloride or NB broth: nutrient broth (Difco, Kansas city, MO), 8.0 g L^{-1}; glucose, 10.0 g L^{-1}; supplemented with Tween 80, 0.2% (pH 6.0–6.2) and kanamycin, 25 μg mL^{-1} when required. Mycobacterial transformants were selected on Middlebrook 7H10 enriched with albumin-dextrose-sodium chloride containing either kanamycin (25 μg mL^{-1}) or hygromycin (10 μg mL^{-1}).

Gene cloning strategies

Standard molecular biology protocols were used for all cloning protocols (Sambrook & Russell, 2001). All PCR products were obtained using thermostable ExTaq polymerase (Takara, Japan) and cloned initially into a TA vector (pGEM-Teasy, Promega, Madison, WI), and then released by digestion with appropriate restriction enzymes before cloning into the final vectors. To facilitate subcloning into expression vectors, restriction enzyme recognition sites were incorporated in the sequence of the primers. The plasmids used in this work are listed in Table 1.

Targeted gene replacement

To perform unmarked deletions in the *ligD* (MSMEG5550), *ku* (MSMEG5560) and *recA* (MSMEG2725) genes of *M. smegmatis*, suicidal recombination delivery vectors were constructed. Each recombination vector contained the 5′ end of the appropriate gene (56 bp-*ligD*; 53 bp-*ku*; 51 bp-*recA*) with upstream regions connected to the 3′ end of the gene (894 bp-*ligD*; 456 bp-*ku*; 539 bp-*recA*) with downstream regions. The 5′ and 3′ fragments of the genes in the resulting vectors were ligated out of frame, resulting in expression of nonfunctional proteins. The various fragments of each gene were amplified using primers listed in Table 2 as follows:

For *ligD*: 5′-end and upstream flanking sequence: D-GR1+D-GR2; 3′-end and downstream flanking sequence: D-GR3+D-GR4.

Table 1. Plasmids used in this study

Plasmid	Description	Source
Cloning vectors		
pGemTEasy	T/A cloning	Promega
PMV306	Mycobacterial integrating vector, Kan^R	Med-Immune Inc., Gaithersburg, MD.
p2NIL	Recombination vector, nonreplicating in mycobacteria, Kan^R	Parish & Stoker (2000)
pGoal17	The source of *Pac*I cassette, Amp^R	Parish & Stoker (2000)
pJam2	Shuttle vector carrying inducible P_{ami} promoter, Kan^R	Triccas *et al*. (1998)
pMV206	Mycobacterial *Escherichia coli* shuttle vector, Kan^R	Med-Immune Inc.
Vectors used for gene replacement		
pMK100	Ku *Bam*HI–*Hin*dIII fragment including 5′ end and its upstream region (1053 bp) in p2NIL, Kan^R	This study
pMK102	Ku *Pst*I–*Hin*dIII fragment including 3′ end and its downstream region (1317 bp) in pMK100, Kan^R	This study
pMK103	pMK102 with *Pac*I cassette from pGoal17, Kan^R	This study
pMK108	ligD *Hin*dIII–*Bam*HI fragment including 5′ end and its upstream region (1973 bp) in p2NIL, Kan^R	This study
pMK110	ligD *Bam*HI–*Kpn*I fragment including 3′ end and its downstream region (1770 bp) in pMK108, Kan^R	This study
pMK111	pMK110 with *Pac*I cassette from pGoal17, Kan^R	This study
pMK120	recA *Pst*I–*Hin*dIII fragment including 5′ end and its upstream region (1305 bp) in p2NIL, Kan^R	This study
pMK121	recA *Kpn*I–*Hin*dIII fragment including 3′ end and its downstream region (1366 bp) in pMK120, Kan^R	This study
pMK122	pMK121 with *Pac*I cassette from pGoal17, Kan^R	This study
Over-production vectors		
pMK112	*ku* under P_{ami} promoter in pJam2, Kan^R	This study
pMK113	*ligD* under P_{ami} promoter in pJam2, Kan^R	This study
pMK116	*ku* in pMV306, Kan^R	This study
pMK117	*ligD* in pMV306, Kan^R	This study
Recircularization assay vectors		
pKSR11	pMV206 carrying gfp in *Bam*HI–*Pst*I sites, Kan^R	M. Rajagopalan (unpublished)
pMV206-hyg	pMV206 carrying Hyg^R gene, Kan^R, Hyg^R	This study

Table 2. Primer sequences used for PCR amplification

Amplified region	Primer name	Primer sequence
Primers used to amplify DNA for targeted gene replacement		
*lig*D 5′ flanking region – sense	D-GR1	5′cccaagcttcgatccgtggggcgtggtg3′
*lig*D 5′ flanking region – reverse	D-GR2	5′cgggatccggattcgtcaggcgaacccgc3′
*lig*D 3′ flanking region – sense	D-GR3	5′cgggatccgccaccgagggttccgtcgcg3′
*lig*D 3′ flanking region – reverse	D-GR4	5′ggggtacctcgtcaccggcgccagcagc3′
ku 5′ flanking region – sense	KuGR1	5′cccaagcttctgtggcccgacgagatcc3′
ku 5′ flanking region – reverse	KuGR2	5′cgggatcccgccttgcggcggatcctcg3′
ku 3′ flanking region – sense	KuGR3	5′aactgcaggcgatgacgtcgggcactgg3′
ku 3′ flanking region – reverse	KuGR4	5′cccaagcttaccggcacgttcaccaggcc3′
*rec*A 5′ flanking region – sense	RecA-GR1	5′aactgcagggcagggttcaccgatcgtg3′
*rec*A 5′ flanking region – reverse	RecA-GR2	5′cccaagcttggccatcgccagttcgagggc3′
*rec*A 3′ flanking region – sense	RecA-GR3	5′cccaagcttgcctgatgagccaggcgctgc3′
*rec*A 3′ flanking region – reverse	RecA-GR4	5′ggggtacctcgagatggtgcggcaaggc3′
Primers used to clone genes for complementation experiments		
*lig*D gene	MsD-s	5′cgggatccgtggcgaggcatccttgggg3′
*lig*D gene	MsD-r	5′gctctagactattcccacacaacctcatcgg3′
ku gene	MsKu-s	5′cggatccatgaaccgtgcggtacgcc3′
ku gene	MsKu-r	5′gctctagactacgacttcttcgcagctgc3′

For the primers used to clone genes for complementation, underlined regions represent additional sequences, which include restriction sites used during cloning steps (*Bam*HI, GGATCC; *Xba*I, TCTAGA).

For *ku*: 5′-end and upstream flanking sequence: KuGR1+KuGR2; 3′-end and downstream flanking sequence: KuGR3+KuGR4.

For *recA*: 5′-end and upstream flanking sequence: RecA-GR1+RecA-GR2; 3′-end and downstream flanking sequence: RecA-GR3+RecA-GR4.

Construction of complementation plasmids

Two *M. smegmatis* genes (*ligD* and *ku*) were PCR amplified (using primers [MsD-s+MsD-r] for *ligD* and [MsKu-s+MsKu-r] for *ku*) and cloned into *Bam*HI–*Xba*I sites of pJam2 vector downstream from the P_{ami} promoter (Table 2). Next, *lig*D and *ku* genes with P_{ami} promoter were excised from these vectors with *Hin*dIII and *Xba*I and cloned into the integration vector pMV306, generating pMK116 and pMK117 for *ku* and *ligD*, respectively.

Disruption of *ku*, *ligD* and *recA* genes

The protocol of Parish & Stoker (2000) was used to disrupt the investigated genes at their native loci on the chromosome. The plasmid DNAs (pMK103, pMK111, pMK122) were treated with NaOH (0.2 mM) and integrated into the *M. smegmatis* mc^2155 chromosome by HR. The resulting single crossover recombinant (SCO) mutant colonies were blue, Kan^R and sensitive to sucrose. The site of recombination was confirmed by PCR and Southern hybridization. The SCO strains were further processed to select for double-crossover (DCO) mutants that were white, Kan^S and resistant to sucrose (2%). As required, further recombination events were used to generate double DCO (dDCO) and triple DCO (tDCO) strains. Analyses by PCR and Southern hybridization were used to distinguish between the wild type and each mutant DCO. Probes to hybridize to each gene were generated by PCR, with labelling by a nonradioactive primer extension system (DIG-labelling system, Amersham, Sweden) as follows: *msligD* = ligDs and ligDr as primers and pMK110 as a template; *msku* = ku-s, ku-r primers and pMK102 as a template; *msrecA* = recA-s, recA-r primers and pMK121 as a template.

Growth in the presence of DNA-damaging agents

Experiments were performed with MMC and *cis*-platinum (*cis*-DDP), chemicals that are known to damage DNA.

Analysis of the effects on growth were performed as follows: NB medium (50 mL) containing 25 ng mL^{-1} of MMC was inoculated with exponentially growing cultures of wild-type and mutant strains to $OD_{600\,nm} = 0.1$ and incubated for 4 h on a shaker at 37 °C. Then the cells were harvested by centrifugation, washed twice with MMC-free medium, suspended in 50 mL of a fresh NB broth and the cultures were grown for a further 45 h on a shaker at 37 °C. To determine the number of dividing cells in each culture, samples were collected and plated every 3 or 6 h. An additional 1–3 mL samples were prepared as described previously (Dziadek *et al.*, 2002) and analysed by a confocal imaging system (Pascal Zeiss Axiovert 200M Microscope, with a Plan Neofluor ×63 oil immersion objective, Zeiss, Germany). Cell length was determined using the Zeiss LSM imaging software, with at least 50 individual cells being measured at each time point.

Analyses of the effects on viability were performed as follows: actively growing cultures ($OD_{600\,nm}$ = 0.6–0.8) were diluted to $OD_{600\,nm}$ = 0.1 and incubated with various concentrations of MMC (0, 50, 150 ng mL^{-1}) or *cis*-DDP (0 and 100 μM) for 4 h. To determine the number of viable cells in each culture, the cells were washed as above, plated on NB plates and grown at 37 °C for 72–96 h.

Transformation with linearized plasmids

Plasmid DNA of *Mycobacterium–Escherichia coli* shuttle vector (pKSR11 or pMV206-hyg) was digested with *Bam*HI or *Eco*RI and *Eco*RV enzymes, purified from an agarose gel and electroporated into *M. smegmatis* wild-type and mutant competent cells. The transformed cells were plated on 7H10-OADC plates with appropriate antibiotic. The quality of electroporated cells was verified with supercoiled DNA of the same vector. The plasmid DNA of transformants was recovered as described previously (Madiraju *et al.*, 2000) and analysed by digestion with restriction enzymes and sequencing.

Results

Disruption of *Mycobacterium smegmatis ku, ligD* and *recA* genes

To allow evaluation of bacterial NHEJ and its interaction with other DNA repair processes, we constructed *Mycobacterium smegmatis* strains defective in production of proteins that are central to NHEJ (LigD and/or Ku) and HR (RecA).

The two-step recombination protocol (Parish & Stoker, 2000) was used to generate unmarked deletions of all three genes within the *M. smegmatis* chromosome. The resultant mutants were verified by PCR and Southern hybridization (Fig. 1). The generation of these strains shows that *ku*, *ligD* and *recA* are not essential genes in *M. smegmatis*, in agreement with previous observations (Frischkorn *et al.*, 1998; Gong *et al.*, 2005). Furthermore, as it was possible to delete simultaneously all genes on the chromosome (Δ*ligD*–Δ*ku*–Δ*recA*), NHEJ- and *recA*-dependent processes are not required when *M. smegmatis* grows in rich media. In such media and in the absence of DNA-damaging agents, there was no noticeable difference in the growth rates of the wild-type cells or those mutated for all three genes studied here (see Fig. 3a).

NHEJ but not *RecA* is essential for re-circularization of incompatible ends in *Mycobacterium smegmatis*

As plasmid DNA carrying *ori*-Al5000 is propagated in mycobacteria in a circular form, the linear form taken up during transformation must be ligated *in vivo* by cellular enzymes before replication proceeds. Use of linearized plasmids with selection markers means that only those cells containing re-circularized plasmids will be viable.

Plasmid DNA of the *Escherichia coli–M. smegmatis* shuttle vector pKSR11 was digested with restriction enzymes in order to produce ends that were compatible (*Bam*HI) or incompatible (*Eco*RI in combination with *Eco*RV). After agarose gel purification, the DNA was transformed by electroporation into various strains of *M. smegmatis* (wild-type, Δ*recA*, Δ*ligD*, Δ*ku*, Δ*ligD*–Δ*ku*). The efficiency of transformation was calculated per 1 μg of DNA and normalized by the number of transformants obtained with supercoiled, control DNA. In all experiments (Fig. 2), the efficiency of transformation with plasmid digested with (*Eco*RI+*Eco*RV) was lower than that obtained with plasmid digested with *Bam*HI, in accordance with expectation that it is more difficult for the cell to process incompatible ends.

The efficiency of transformation for theδ*recA* strain and the control wild-type strain was very similar (Fig. 2). By contrast, *M. smegmatis* strains with deletions in the NHEJ genes (Δ*lig*D, Δ*ku* and Δ*ku*-Δ*lig*D) were transformed with 83–96% lower efficiency for *Bam*HI-digested plasmid. For strains lacking functional NHEJ, it was generally not possible to obtain transformants with plasmid digested with (*Eco*RI+*Eco*RV).

Two additional control strains were prepared that allowed conditional complementation of the *ligD* and *ku* deletions with additional copies of the relevant wild-type gene. The *ligD* and *ku* under control of P_{ami} were introduced into the *att*B site with integration plasmids pMK116 and pMK117. The strains were transformed with linearized-plasmid [pMV206-Hyg digested with *Bam*HI and (*Eco*RI+*Eco*RV)], as described above. In all experiments, complementation of the mutant strains with the appropriate deleted gene returned the efficiency of transformation to the level of the wild-type strain (Fig. 2).

Thus, the presence of NHEJ proteins increased the efficiency of transformation by *Bam*HI linearized plasmids and allowed the self-ligation of plasmids linearized by *Eco*RI+*Eco*RV. On the other hand, the presence of RecA is likely to be not significant for self-ligation of linearized plasmid DNA.

An intact *recA* gene, but not *ligD* and *ku*, is essential to repair DNA damage induced by MMC

Mitomycin C is a DNA-damaging agent, which induces mainly interstrand DNA cross-links, although processing of the damage is also likely to lead to the formation of DSBs (Dronkert & Kanaar, 2001; Khil & Camerini-Otero, 2002; Niedernhofer *et al.*, 2004). Importantly, NHEJ is used to

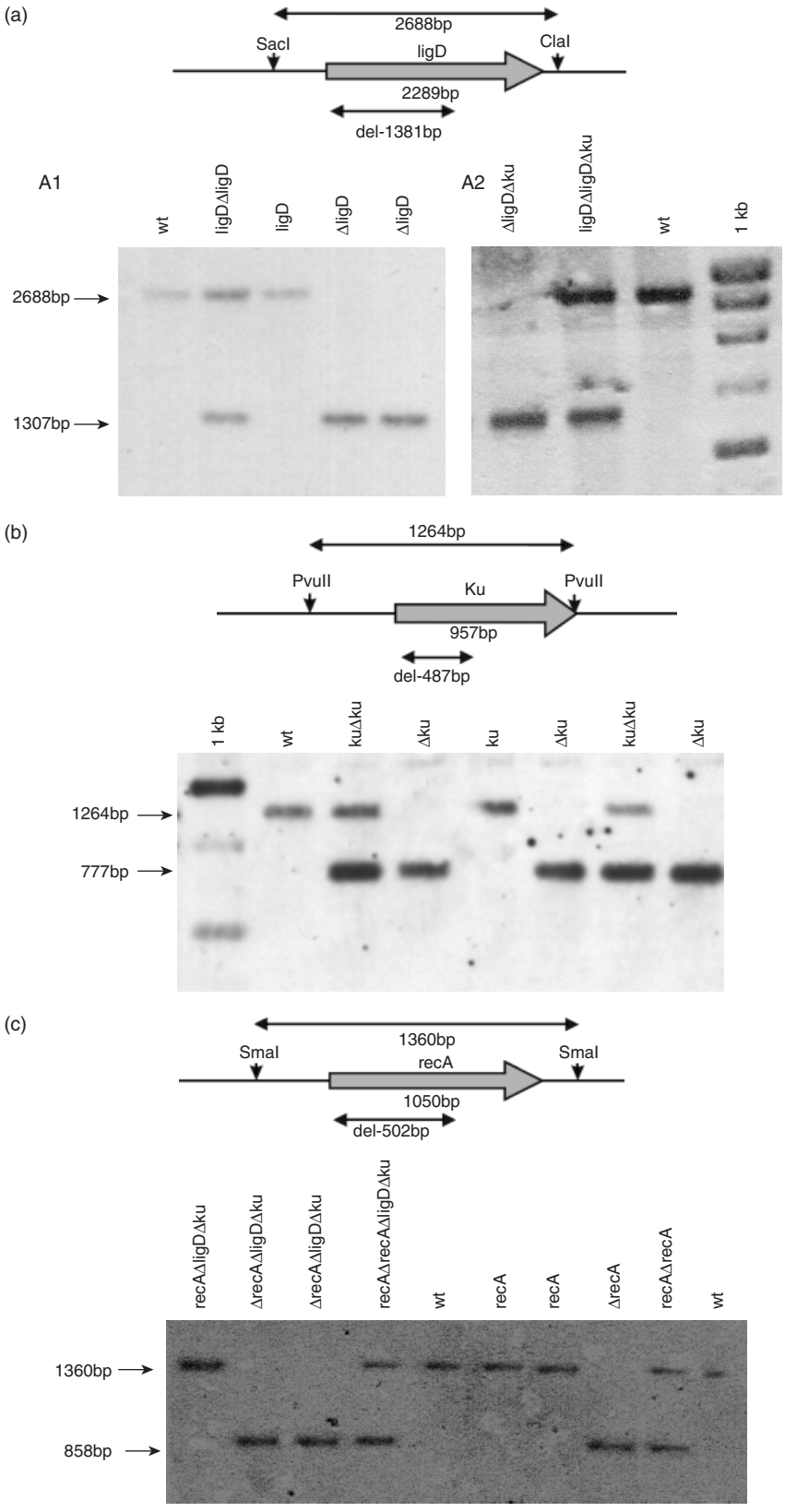

Fig. 1. Replacement of *ligD*, *ku* and *recA* genes of *Mycobacterium smegmatis* with mutated genes. The chromosomal localization of each gene is represented by a grey arrow. Relevant restriction enzyme sites and the sizes of fragments obtained with the wild-type and deleted genes are denoted. A 1 kb ladder acted as a marker for each gel. (a) DNA was digested with *Sac*I and *Cla*I. (a1) Southern analysis of genomic DNA isolated from wild-type *M. smegmatis* (wt) and recombinants: ligDΔligD [*M. smegmatis ligDΔligD* obtained by single crossover (SCO) recombination of the wild-type strain with plasmid DNA pMK111]; ligD (*M. smegmatis ligD* obtained from *M. smegmatis ligDΔligD*); ΔligD (two constructs of *M. smegmatis* Δ*ligD*). (a2) Southern analysis of genomic DNA isolated from wild-type *M. smegmatis* (wt) and recombinants: ligDΔligDΔku (*M. smegmatis ligDΔligDΔku* obtained by SCO recombination of the *M. smegmatis* Δ*ku* with plasmid DNA pMK111); ΔligDΔku (*M. smegmatis* Δ*ligD*Δ*ku*). The DNA was identified by hybridization to a *lig*D-probe. The expected hybridization bands for the wild-type and mutated genes (2688 and 1307 bp, respectively) are indicated by arrows. (b) DNA was digested with *Pvu*II. Southern analysis of genomic DNA isolated from wild-type *M. smegmatis* (wt) and recombinants: two constructs of kuΔku (*M. smegmatis kuΔku* obtained by SCO recombination of the wild-type strain with plasmid pMK103); ku (*M. smegmatis ku* obtained from *M. smegmatis kuΔku*); three constructs of Δku (*M. smegmatis* Δ*ku*). The DNA was identified by hybridization to a *ku*-probe. The expected hybridization bands for the wild-type and mutated genes (1264 and 777 bp, respectively) are indicated by arrows. (c) DNA was digested with *Sma*I. Southern analysis of genomic DNA isolated from wild-type *M. smegmatis* (wt) and recombinants: recAΔrecA (*M. smegmatis recAΔrecA* obtained by SCO recombination of the wild-type strain with plasmid pMK122); recAΔrecAΔligDΔku (*M. smegmatis recAΔrecAΔligDΔku* obtained by SCO recombination of the *M. smegmatis* Δ*ligD*Δ*ku* with plasmid pMK122); recA (*M. smegmatis recA* obtained from *M. smegmatis recAΔrecA*); recAΔligDΔku (*M. smegmatis recAΔligDΔku* obtained from *M. smegmatis recAΔrecAΔligDΔku*); ΔrecA (*M. smegmatis* Δ*recA*); two constructs of ΔrecAΔligDΔku (*M. smegmatis* Δ*lig*DΔ*ku*Δ*rec*A). The DNA was identified by hybridization to a Δ*rec*A-probe. The expected hybridization bands for the wild-type and mutated genes (1360 and 858 bp, respectively) are indicated by arrows.

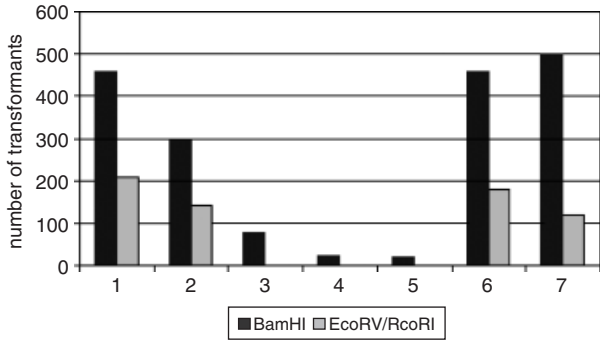

Fig. 2. Double-strand breaks in plasmids are repaired mainly by non-homologous end joining. The efficiency of transformation with 0.5 μg of linear plasmid DNA containing *Bam*HI cohesive ends (black bar) or *Eco*RI/*Eco*RV incompatible ends (grey bar) was measured. All experiments were performed in strains derived from *Mycobacterium smegmatis* mc^2155 as follows (*x*-axis): 1, wild type; 2, Δ*recA*; 3, Δ*ligD*; 4, Δ*ku*; 5, Δ*lig*DΔ*ku*; 6, Δ*lig*D-P_{ami}*lig*D; 7, Δ*ku*-P_{ami}*ku*. Each transformation experiment was repeated three to five times with freshly prepared DNA and competent cells, with the representative result being presented in the figure. The *y*-axis represents the number of transformants obtained, calculated for 1 μg of linear DNA.

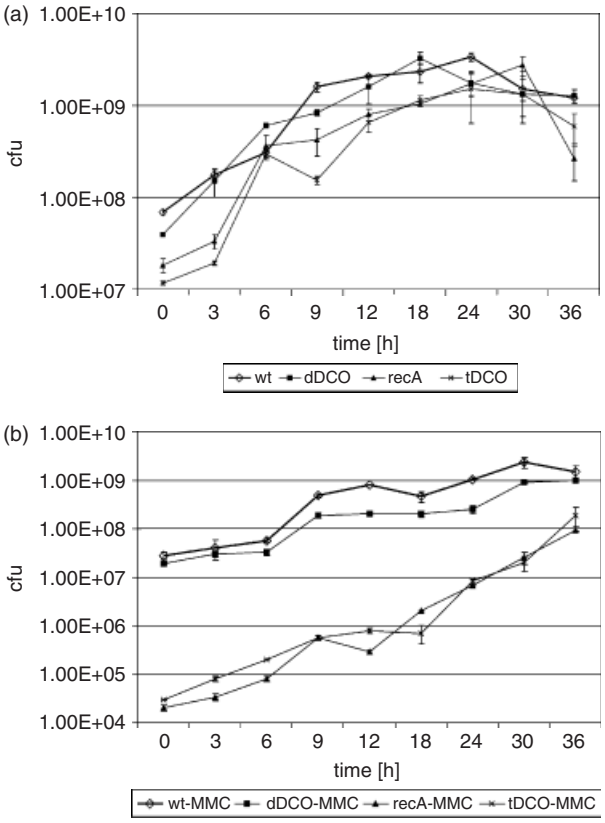

Fig. 3. Growth analysis of *Mycobacterium smegmatis* wild-type and RecA/nonhomologous end joining (NHEJ)-defective strains exposed (b) or not (a) to mitomycin C (MMC). The exponentially growing cultures of wild-type and mutant strains were diluted to $OD_{600\,nm} = 0.1$. A half of each diluted culture was supplemented with 25 ng mL^{-1} of MMC and incubated for 4 h. The remaining half of each culture was incubated without MMC for the same period of time. Next, cells were harvested and suspended in the fresh MMC-free medium and incubated for 36 h. (a) represents 36 h incubation time of cells that were not exposed to MMC. (b) represents 36 h incubation time of cells that were previously exposed to MMC. Zero (0) on the *x*-axis indicates time when medium of each culture [containing MMC – (b) or not (a)] was replaced with fresh MMC-free medium. The numbers of viable cells were determined by counting the number of colony forming units (cfu). Strain references are as follows: a – *M. smegmatis* wild type (wt); *M. smegmatis* ΔligDΔku (dDCO); *M. smegmatis* ΔrecA (recA-); *M. smegmatis* ΔrecAligDΔku (tDCO); b – *M. smegmatis* wild type treated with MMC, (wt-MMC); *M. smegmatis* ΔligDΔku treated with MMC (dDCO-MMC); *M. smegmatis* ΔrecA treated with MMC (recA-MMC); *M. smegmatis* ΔrecAligDΔku treated with MMC (tDCO-MMC). The standard deviation was calculated from four independent experiments.

repair damage induced by MMC in yeast and mammalian cells, albeit in a cell cycle-dependent manner (De Silva *et al*., 2000; McHugh *et al*., 2000). In the light of these previous observations, we wondered if NHEJ may play any role in the response of *M. smegmatis* to damage induced by MMC.

All strains constructed in this study were tested for their ability to grow with and without exposure to MMC. In the absence of DNA-damaging agents, the growth rates of the wild-type cells and those mutated for all three genes studied here were similar (Fig. 3a). Incubation of *M. smegmatis* cells with MMC decreased dramatically the viability of the *recA*-deletion strains (*M. smegmatis* Δ*recA*; Δ*recA*–Δ*ligD*–Δ*ku*) in comparison with wild-type and NHEJ-defective strains (Fig. 3b). NHEJ-defective and wild-type strains incubated with MMC had the same viability for the first 6 h after MMC treatment, but during further incubation wild-type cells grew faster than the NHEJ-defective mutants (see 9–30 h time points in Fig. 3b). No significant differences were observed in the number of viable cells for the Δ*recA* mutant and triple-mutant (Δ*recA*–Δ*ligD*–Δ*ku*). This data suggests that the repair of DNA damaged with MMC is coordinated mainly by *recA*-dependent processes, with NHEJ playing a relatively minor role in such genome repair.

The observed increase in the number of RecA-defective cells after exposure to MMC could be due to the repair of DNA by RecA/NHEJ independent pathway(s) or could be due to a small population of cells that are undamaged by the MMC. To differentiate between these possibilities we increased the concentration of MMC and analysed the viability of the cells (data not shown). The highest concentration of MMC was lethal for all Δ*recA* mutants, suggesting that a RecA-dependent mechanism is essential for the repair of DNA damaged with MMC. Treatment with another mutagen, *cis*-DDP, produced similar observations. *Cis*-DDP at 500 μM did not significantly decrease the viability of the wild-type strain or the Δ*ligD*–Δ*ku* mutant, but the viability of Δ*recA* strains decreased by 6-logs (data not shown).

Table 3. Filamentation of cells resulting from inhibition of cell division

	Time (h)											
	0			12			24			45		
	Cells length			Cells length			Cells length			Cells length		
Strain	Average size	≥8 μm	≥10 μm	Average size	≥8 μm	≥10 μm	Average size	≥8 μm	≥10 μm	Average size	≥8 μm	≥10 μm
mc2	4.402	1 (2%)	1 (2%)	3.967	0	0	2.984	0	0	2.893	0	0
Mc2-MMC	5.966	7 (14%)	1 (2%)	4.359	2 (4%)	1 (2%)	3.075	3 (6%)	1 (2%)	3.3	2 (4%)	2 (4%)
dDCO	4.303	0	0	3.823	0	0	2.559	1 (2%)	1 (2%)	2.443	0	0
dDCO-MMC	6.748	13 (26%)	7 (14%)	5.403	7 (14%)	2 (4%)	3.578	1 (2%)	1 (2%)	3.606	2 (4%)	2 (4%)
RecA	4.561	1 (2%)	0	4.197	1 (2%)	0	3.716	0	0	3.51	0	0
RecA-MMC	5.975	5 (10%)	1 (2%)	8.139	16 (32%)	10 (20%)	5.15	5 (10%)	2 (4%)	4.082	3 (6%)	1 (2%)
T	4.693	1 (2%)	1 (2%)	5.069	2 (4%)	0	4.86	3 (6%)	2 (4%)	3.973	4 (8%)	2 (4%)
T-MMC	6.619	11 (22%)	3 (6%)	6.529	8 (16%)	5 (10%)	6.614	12 (24%)	6 (12%)	4.216	4 (8%)	4 (8%)

See Materials and methods for a detailed description of this experiment.

Time – incubation time (h) after MMC was washed out.

Strain references are as follows: dDCO, Δ*ligD*Δ*ku*; mc2, *Mycobacterium smegmatis* wild type; RecA, *Mycobacterium smegmatis* Δ*rec*A; T, *Mycobacterium smegmatis* Δ*ligD*Δ*ku*Δ*recA*.

MMC – cells were incubated in 25 ng mL^{-1} MMC before changing to fresh media.

At least 50 cells were measured at each time point.

Influence of *recA*-dependent processes and NHEJ on cell division after treatment with MMC

Bacterial cells exposed to MMC have inhibition of replication and cell division (Kawai *et al*., 2003). It is generally accepted that cells that cannot divide continue to grow and elongate (Dziadek *et al*., 2002, 2003). Upon repair of DNA damage, cells should return to the normal cell cycle, with a consequent decrease in cell length upon completion of cell division. To assess if the effects of MMC on the mycobacterial cell cycle were influenced by RecA- or NHEJ-dependent processes, the morphology of cells was monitored after DNA damage with MMC. The *M. smegmatis* wild-type strain and all mutated strains generated during this study were grown in media with and without MMC, as described above. The DNA damage caused by MMC will block DNA replication, which will inhibit cell division. To determine the extent of inhibition of cell division, cell length was measured immediately after treatment with MMC and 12, 24 and 45 h later (Table 3). Immediately after treatment with MMC, all mutant strains had a larger average size than the wild type, but this was particularly pronounced for the double mutant (Δ*ligD*–Δ*ku*) and triple mutant (Δ*ligD*–Δ*ku*–Δ*recA*). Interestingly, for Δ*recA* strains there was a delay in the return of cell length to that before MMC treatment, suggesting that intact RecA is required for the optimal response of mycobacterial cells to DNA damage from MMC. At longer times of growth, all mutant strains were more sensitive to MMC than the wild type, as indicated by the presence of more elongated cells. Moreover, mutants with defective NHEJ- and RecA-dependent pathways were the most sensitive. Thus, there is qualitative agreement between this data and that observed in the growth experiments, displaying the major role of RecA-dependent pathways in repairing DNA damaged with MMC, although a minor role of the NHEJ pathway is also detectable.

Discussion

Previous studies have identified that functional NHEJ proteins are encoded by the genomes of some mycobacteria, including *Mycobacterium smegmatis* (Weller *et al*., 2002; Della *et al*., 2004; Gong *et al*., 2004, 2005). To assess the roles of NHEJ in bacterial DNA repair, especially in its relationship with RecA-dependent processes, we constructed *M. smegmatis* strains that were defective in the production of proteins central to NHEJ (LigD and/or Ku), RecA-dependent processes and both systems. As it was possible to obtain strains with deletions in all three of the relevant chromosomal genes (Δ*ligD*–Δ*ku*–Δ*recA*), NHEJ- and RecA-dependent processes are not essential when *M. smegmatis* grows in rich media. This is consistent with previous findings with *Bacillus subtilis* (Weller *et al*., 2002). Use of several different assays of DNA repair capacity identified that both NHEJ and RecA-dependent processes could bring about repair of the damage, but found little evidence for synergistic interactions between these two processes. For example, the repair of DNA damaged with MMC or *cis*-DDP was coordinated mainly by RecA-dependent processes, with NHEJ playing a relatively minor role. Conversely, in an assay that is typically used to assess NHEJ capability, the efficiency

of transformation of *M. smegmatis* by linearized plasmids was dramatically reduced in the absence of functional NHEJ proteins, whereas inactivation of *recA* had no significant effect.

To evaluate the relationship of RecA-dependent pathways and NHEJ in bacterial DNA repair systems, we used the DNA-damaging agent MMC. This antitumour compound forms adducts in DNA including ICLs that are extremely toxic (Dronkert & Kanaar, 2001). These adducts inhibit separation of DNA strands, blocking transcription or replication of DNA. Although the mechanism of repair of such damage is not completely clear, stalled replication forks can result in fork regression and formation of DSBs within DNA (De Silva *et al*., 2000; McHugh *et al*., 2000; Dronkert & Kanaar, 2001; Niedernhofer *et al*., 2004). In *Escherichia coli* and *Saccharomyces cerevisiae*, efficient repair of ICLs requires intact NER and HR systems, for which RecA activity is important (Cole & Sinden, 1975; Jachymczyk *et al*., 1981). Using the various mutants constructed here, it is clear that RecA-dependent processes are the primary mechanism in mycobacteria to repair DNA damaged with MMC. The *M. smegmatis* Δ*rec*A strain viability was decreased by 99.9% in 4 h with 25 ng mL^{-1} of MMC, and twice this concentration was found to be lethal for these cells. Upon exposure to 25 ng mL^{-1} of MMC the wild-type strain and NHEJ defective mutants viability decreased by only 50%, although monitoring of the number of viable cells in these strains revealed a slow-down in the doubling time of NHEJ-defective mutants (Fig. 2b). As this effect was observed in the three different strains mutated for NHEJ, repeated in three to five independent experiments, it suggests a minor but detectable role of mycobacterial NHEJ in the repair of DNA damaged by MMC. These observations were confirmed by the analysis of the morphology of the cells. The bacterial cells defective in division became elongated, in a manner similar to that described for FtsA-Z conditional mutants (for reviews, see Margolin, 2000; Errington, 2003). MMC treatment damages DNA, affects replication, eventually leading to inhibition of cell division. Thus, cells with extensive DNA damage should be longer than dividing, undamaged cells. Compared with wild-type strains, the NHEJ-defective strains contained more elongated cells for up to 12 h after treatment with MMC, confirming that these strains are not as effective as the wild-type strain in their repair of damage induced by MMC. It is interesting that elongation of Δ*rec*A cells treated with MMC were observed for longer periods than compared with wild-type strains or those defective only in NHEJ. This indicates that intact RecA protein is important for mycobacteria to recover from DNA damage induced by MMC, consistent with a central role of intact RecA for returning cells to their normal cell cycle after DNA damage (Lusetti & Cox, 2002). The natural mutation rate of stationary phase is higher compared with the logarithmic phase of growth because of accumulation of natural metabolites, which can be mutagenic to DNA. We observed that a small population of cells defective in both RecA and NHEJ were elongated in stationary phase ($OD_{600\,nm} = 2.5$) even without previous contact with MMC. Such cells were not observed in stationary cultures of wild-type strain or mutants defective in only RecA or NHEJ. Thus, the absence of both RecA and NHEJ leads to increased problems in relation to the repair of spontaneous damage, although the reasons behind this are unclear and the comprehension of the specific role of NHEJ warrants further investigation.

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