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ORIGINAL PAPER

The *Deinococcus radiodurans* SMC protein is dispensable for cell viability yet plays a role in DNA folding

Claire Bouthier de la Tour · Magali Toueille · Edmond Jolivet · Hong-Ha Nguyen · Pascale Servant · Françoise Vannier · Suzanne Sommer

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Abstract *Deinococcus radiodurans* contains a highly condensed nucleoid that remains to be unaltered following the exposure to high doses of γ-irradiation. Proteins belonging to the structural maintenance of chromosome protein (SMC) family are present in all organisms and were shown to be involved in chromosome condensation, pairing, and/or segregation. Here, we have inactivated the *smc* gene in the radioresistant bacterium *D. radiodurans*, and, unexpectedly, found that *smc* null mutants showed no discernible phenotype except an increased sensitivity to gyrase inhibitors suggesting a role of SMC in DNA folding. A defect in the SMC-like SbcC protein exacerbated the sensitivity to gyrase inhibitors of cells devoid of SMC. We also showed that the *D. radiodurans* SMC protein forms discrete foci at the periphery of the nucleoid suggesting that SMC could locally condense DNA. The phenotype of *smc* null mutant leads us to speculate that other, not yet identified, proteins drive the compact organization of the *D. radiodurans* nucleoid.

Keywords *Deinococcus radiodurans* · SMC · Nucleoid compaction · Resistance to ionizing radiation · Gyrase inhibitors · SbcCD complex

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C. Bouthier de la Tour · M. Toueille · E. Jolivet · H.-H. Nguyen · P. Servant · F. Vannier · S. Sommer (✉)
Univ Paris-Sud, CNRS UMR8621, CEA LRC42V, Institut de Génétique et Microbiologie, Bâtiment 409, 91405 Orsay Cedex, France
e-mail: suzanne.sommer@igmors.u-psud.fr

Introduction

Deinococcus radiodurans belongs to a family of bacteria characterized by an exceptional capacity to cope with the lethal effects of DNA-damaging agents, including ionizing radiation, UV light and desiccation. Its radioresistance is linked to its extraordinary ability to reconstruct a functional genome from hundreds of radiation-induced chromosomal fragments, whereas the genome of most organisms is irreversibly shattered under the same conditions. Active (DNA-repair processes) and passive (nucleoid organization) mechanisms are probably intimately combined to enable its survival after ionizing radiation [for review, see (Blasius et al. 2008; Cox and Battista 2005]. The *D. radiodurans* nucleoids adopt a condensed ring-like structure that remains to be unaltered after the exposure to high doses of γ-irradiation (Levin-Zaidman et al. 2003; Zimmerman and Battista 2005). The tightly packed structure of the nucleoid appears as a common feature among radioresistant bacteria (Zimmerman and Battista 2005) suggesting that it may play an important role in DNA double-strand break repair by limiting the diffusion of the DNA fragments and holding together free-DNA ends. However, the mechanisms by which *D. radiodurans* chromosomes are organized and compacted are poorly understood. Only four homologs of the 12 nucleoid-associated proteins identified in *Escherichia coli* can be found in the *D. radiodurans* genome. This suggests either the existence of as yet unknown DNA compacting factors, or special functions of the classical proteins involved in nucleoid organization. Among the proteins involved in DNA compaction, the structural maintenance of chromosome (SMC) protein might play a critical role in *D. radiodurans*.

zStructural maintenance of chromosome proteins possesses an ATP-dependent DNA compacting activity. They are conserved in all domains of life (Losada and Hirano

2005) and are required for many aspects of chromosome dynamics (for review, see (Strunnikov 2006) including chromosome condensation, sister chromatin cohesion, chromosome partition, DNA repair and recombination. In bacteria, SMC proteins from *Bacillus subtilis*, *E. coli* and *Caulobacter crescentus* have been extensively studied and appear to have similar functions. The SMC proteins are not absolutely essential for survival, but the disruption of the corresponding genes leads in many, but not all, bacterial species to severely retarded growth, temperature-sensitive growth and defects in chromosome segregation and condensation (Britton et al. 1998; Graumann et al. 1998; Jensen and Shapiro 1999, 2003). The cellular functions of SMC proteins appear tightly linked to the level of DNA supercoiling. Indeed, the phenotypes of *smc* deletion mutants in *E. coli* and *B. subtilis* are partially suppressed by an increase in chromosomal supercoiling following mutations in the gene-encoding topoisomerase I (Holmes and Cozzarelli 2000; Sawitzke and Austin 2000). Moreover, the depletion of SMC in these two species renders the cells hypersensitive to drugs that inhibit DNA gyrase activity (Adachi and Hiraga 2003; Lindow et al. 2002a; Sawitzke and Austin 2000). SMC proteins were shown to be associated with the nucleoid. In *B. subtilis*, SMC localizes to discrete foci (Lindow et al. 2002b) and appears to interact with different regions on the chromosome during the cell cycle (Volkov et al. 2003). The *E. coli* MukB (SMC-related protein) also appears to be associated with the nucleoid (den Blaauwen et al. 2001), forming one to two foci per nucleoid (Danilova et al. 2007).

A homolog of the *B. subtilis smc* gene, *DR1471*, has been identified in *D. radiodurans* (Makarova et al. 2001; White et al. 1999). Here, we have disrupted the Deinococcal *smc* gene and analyzed the effects of SMC depletion in *D. radiodurans*. Unexpectedly, we found that the *smc* null mutant has no discernible phenotype, except for hypersensitivity to novobiocin and nalidixic acid. Interestingly, a defect in the SMC-like SbcC protein (Connelly et al. 1998) exacerbated the sensitivity to gyrase inhibitors of cells devoid of SMC. As in *B. subtilis*, the *D. radiodurans* SMC protein formed discrete foci at the periphery of the nucleoid, suggesting that SMC protein could locally condense DNA. According to our data, *D. radiodurans* SMC protein is likely involved in DNA folding but other, not yet identified, proteins might play a major role in driving the compact organization of the nucleoid.

Materials and methods

Materials, media, bacterial strains and plasmids

All reagents, materials and media were from previously reported sources (Bonacossa de Almeida et al. 2002). When necessary, media were supplemented with the appropriate antibiotics used at the following final concentrations: kanamycin 6 μg ml^{-1}, chloramphenicol 3.5 μg ml^{-1} and spectinomycin 75 μg ml^{-1}.

Bacterial strains are listed in Table 1. The *smc* null GY12428 and the SPA-tagged *smc* GY12820 mutants were constructed by the tripartite ligation method (Mennecier et al. 2004). To construct the GY12428 strain, a cassette containing the resistance gene to chloramphenicol was ligated to the chromosomal sequences 500 bp upstream and downstream of the coding of the *smc* gene. To construct the GY12820 strain, a cassette containing the SPA tag (Zeghouf et al. 2004) and the resistance gene to chloramphenicol was ligated to the C-terminal region of the *smc* gene and to the chromosomal region directly downstream the gene. The mutated alleles constructed in vitro were used to transform *D. radiodurans* to replace their wild-type counterparts by homologous recombination. The Δ*smc*Ω*cat* Δ*sbcCD*Ω*kan* double mutant was constructed by transformation of the Δ*smc* GY12428 by genomic DNA of the Δ*sbcCD* GY12910 strain (Bentchikou et al. 2007). Strain GY13777 is a wild-type strain containing a second copy of the *smc* gene cloned onto the thermosensitive replication vector p13480 under the control of a P_{Spac} promoter. The plasmidic *smc* gene is expressed constitutively in strain GY13777, devoid of the LacI repressor. To inactivate the chromosomal copy of *smc*, the chromosomal DNA from the Δ*smc* mutant strain GY12428 was used to transform GY13777. The resulting strain, GY13787, was used to test the non-essentiality of *smc*. The genetic structure of all the mutants was verified by PCR. Oligonucleotides used for strain construction and diagnostic PCR tests will be provided on request.

Plasmids are listed in Table 1. Plasmid p11975, used to analyze the topoisomer distribution, is a shorter derivative of the p11520 shuttle vector (Bentchikou et al. 2007) obtained by circularization of the 6,749 bp *Hin*dIII fragment of p11520, in which the part encoding the replicon active in *E. coli* was deleted. Plasmid p12723 was the source of the SPA-Tag *cat* cassette used in the construction of the *smc*-SPA-tagged allele. The SPA-Tag was amplified using plasmid pMZ3F (Zeghouf et al. 2004) as template. The SPA-Tag and the P_{tufA}-*cat* cassette were sequentially cloned into the pGEMT Easy vector (Promega). Plasmid p13407, used to express the SPA-tagged *smc* gene under the control of a P_{Spac} promoter, was constructed by cloning the *smc-spa*-tagged gene amplified by PCR from the genomic DNA of strain GY12820 into the expression shuttle vector p11559 (Mennecier et al. 2004) between the *Nde*I and the *Xho*I sites. The expression of SMC–SPA protein was induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) to the media. Plasmid p13848 used for conditional inactivation of SMC was constructed by

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or other relevant characteristics	Source or references
Strains		
E. coli		
DH5α	*supE44 hsdR17 recA1 endA1 lacZΔ M15*	Laboratory stock
SCS110	*endA dam dcm supE44* Δ(*lac-proAB*) (*F'traD36 proAB lacI^{q}ZΔ M15*)	Laboratory stock
D. radiodurans		
R1	ATCC 13939	(Anderson et al. 1956)
GY12428	As R1 but Δ*smc* Ω *cat*	This work
GY12910	As R1 but Δ*sbcCD* Ω *kan*	(Bentchikou et al. 2007)
GY13316	As GY12428 but *sbcCD* Ω *kan*	This work
GY11795	R1 (p11975)	This work
GY13323	GY12428 (p11975)	This work
GY13324	GY12910 (p11975)	This work
GY13326	GY13316 (p11975)	This work
GY12820	As R1 but *smc::spa* Ω *cat*	This work
GY13335	GY12428 (p13407)	This work
GY13777	R1 (p13848)	This work
GY13781	R1 (p13840)	This work
GY13785	R1 (p11554)	This work
GY13787	Δ*smc* Ω *cat* (p13848)	This work
Plasmids		
P11520	Shuttle vector *E. coli–D. radiodurans;* Spc^R	(Mennecier et al. 2004)
pGTC101	Source of the P$_{tufA}$::*cat* cassette	(Earl et al. 2002)
pMZ3F	Source of the *spa* Tag	(Zeghouf et al. 2004)
p11975	Deletion derivative of p11520; replicates only in *D. radiodurans*	This work
p13407	p11559; P$_{Spac}$::*smc::spa*	This work
p11559	Expression vector; P$_{Spac}$, P$_{tufA}$::*lacI*, Spc^R	(Lecointe et al. 2004)
pGEMT Easy	Cloning vector	Promega
p12723	pGEMT; *spa-tag*, P$_{tufA}$::*cat*	This work
p13840	Thermosensitive replication vector *repU$_{Ts}$*; Spc^R	(Nguyen et al. 2009)
P11554	Shuttle vector *E. coli-D. radiodurans*; Spc^R	Laboratory stock
P13848	p13840; P$_{Spac}$::*smc*	This work

cloning the *smc* gene under the control of the P$_{Spac}$ promoter onto the thermosensitive replication vector p13480 (Nguyen et al. 2009).

Assay of the level of supercoiling of plasmid DNA

Plasmid p11975 was purified using a variant of the alkaline lysis method. Cells from a 10-ml culture of plasmid-containing strains at an $OD_{650} = 0.7$ were collected by centrifugation and resuspended in 400 µl of a solution containing equal volumes of 0.5 M EDTA and butanol saturated in 0.5 M EDTA. The suspension was stirred for 15 min at room temperature, centrifuged 2 min at 15,000*g* and the pellet resuspended in 200 µl of 0.5 M EDTA. After incubation for 45 min at 70°C, the butanol-stripped cells were harvested by centrifugation, washed twice in 400 µl of buffer A (10 mM Tris–HCL pH 8, 5 mM EDTA, 0.5 M NaCl) and resuspended in 100 µl of buffer A supplemented

with lysozyme (5 mg ml^{-1}). The suspension was incubated at 37°C for 60 min. Then, 400 µl of a solution containing 10 mM Tris–HCl pH 8, 10 mM EDTA, 0.5% SDS supplemented with proteinase K at 4 mg ml^{-1} and RNase at 50 µg ml^{-1}, were added and incubated at 56°C for 3 h. After this step, the Qiaprep kit protocol of plasmid purification was followed. Plasmid DNA was subjected to electrophoresis on 1.8% agarose gels containing chloroquine at 15 µg ml^{-1} in TPE buffer (36 mM Tris–HCl, 30 mM NaH_2PO_4, 1 mM EDTA) at 2 V/cm for 20 h with the recycling of chloroquine-containing running buffer. Then, the gel was washed in H_2O for 2 h and stained with 1 µg ml^{-1} of ethidium bromide.

Treatment of cells with gamma irradiation

Cells grown exponentially in TGY2X ($OD_{650} = 0.5$) were concentrated 20 times and irradiated on ice with a ^{137}Cs irradiation system (Institut Curie, Orsay, France) at a dose rate of 41.7 Gy min^{-1}. Following irradiation, diluted samples were plated on TGY2X, and incubated at 30°C for 3–4 days before the colonies were counted.

Assay of sensitivity to DNA gyrase inhibitors

Cultures of exponentially growing cells at $OD_{650} = 0.5$ were serially diluted and aliquots (10 µl) of each dilution were spotted on TGY2X agar or TGY2X agar supplemented with 20 ng ml^{-1} of novobiocin or 40 µg ml^{-1} of nalidixic acid. Plates were incubated at 30°C for 3–5 days and 1 mM IPTG was added to the culture media when GY13335 strain was used.

Assay of essentiality of the *smc* gene

Cultures of strain GY13877 grown at 28°C in liquid medium with spectinomycin were serially diluted and aliquots (10 µl) of each dilution were spotted on TGY agar with or without spectinomycin. The plates were incubated at 28 or 37°C for 3–4 days.

Fluorescence microscopy

Cells were grown in TGY2X to an $OD_{650} = 0.5$. Aliquots (1 ml) were removed and the cells were fixed using toluene at 3% final concentration. Cell membranes were stained with *N*-(3-triethylammonium-propyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM 4-64) at 10 µg ml^{-1} and the nucleoid with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) at 2 µg ml^{-1}. FM 4-64 stains the lipid membranes with red fluorescence (excitation/emission 515/640 nm) and DAPI stains the nucleoid with blue fluorescence (excitation/emission 350/470 nm). The stained cells were observed using a Leica DM RXA microscope. Images were captured with a CDD camera 5 MHz Micromax 1300Y (Roper Instruments). The final reconstructed images were obtained by deconvoluting Z-series with metamorph software (Universal Imaging).

Western blot analysis of SPA-tagged SMC protein

A 10 ml cultures at an $OD_{650} = 0.5$ were centrifuged, the pellets resuspended in 200 µl of SSC1× buffer and the cells disrupted with a FastPrep instrument (FP120, Bio101) using 0.1 g of glass beads (500 µm) and four pulses of 30 s. Cell debris were removed by centrifugation at 20,000*g* for 10 min at 4°C and the supernatant constituted the cell extract. The protein concentrations were measured with a protein assay kit (Biorad) using the manufacturer's micro-assay procedure. Aliquots of the cell extracts were subjected to electrophoresis through a 12% SDS-polyacrylamide gel and the proteins were transferred on to a polyvinylidene difluoride (PVDF) membrane (Amersham) and treated with a 1:5,000 dilution of monoclonal mouse anti-Flag antibodies (Sigma-Aldrich). Following the incubation with a secondary peroxidase-labeled anti-mouse antibody, the antigen–antibody complex was visualized using an ECL kit (Amersham). The estimated size of SPA-tagged SMC protein is 126 kDa.

Immunofluorescence labeling

A 0.4 ml aliquots of exponential phase cultures ($OD_{650} = 0.25$) of strains expressing the SMC–SPA tagged protein were fixed by the addition of paraformaldehyde at a final concentration of 4% in the culture medium and incubated for 2 h at 4°C. The cells were then centrifuged and the pellet washed in 1× PBS. In order to permeabilize the cell envelope, the cells were treated with 2 mg ml^{-1} lysozyme for 30 min at 37°C followed by incubation with 0.1% triton X-100 in PBS for 5 min at room temperature. Finally, the cells were washed in PBS and resuspended in 25 µl of PBS. A 3 µl aliquot was applied to a poly-L-lysine pretreated slide spot, allowed to air dry and fixed by incubating in 4% PFA for 20 min at 37°C. Cells were then blocked in 2% BSA in PBS-T (0.05% Tween 20 in PBS) and incubated for 2 h at 37°C with a monoclonal mouse anti-Flag antibody (Sigma-Aldrich) diluted 1/700 in blocking solution. After 20 min of washing in PBS-T, the cells were incubated for 1 h at 37°C with an FITC-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) diluted 1/250 in blocking solution and washed for 20 min in PBS-T. Cells were finally stained with DAPI 10 µg ml^{-1} for 10 min at room temperature. After a final wash in PBS-T, slides were mounted using fluoromount G as a mounting medium (Fluoprobes). Image

acquisition and treatment were performed as described above.

Results

Identification of the *D. radiodurans* smc homolog

The putative *smc* gene from *D. radiodurans* (*DR1471*) encodes a protein of 1,100 amino acids with a calculated molecular weight of 118 kDa. The similarities of the bacterial SMC proteins are specifically located in three SMC signature motifs: the N-terminal and the C-terminal domains, each corresponding to a one and a half ATPase domain, and the central region of the protein corresponding to the hinge region of SMC proteins (Strunnikov 2006). The N-terminal (aa 9–181), C-terminal (aa 982–1,098), and central (aa 465–722) regions of DR1471 exhibit 36, 41 and 30% amino acid identity with the corresponding regions of *B. subtilis* SMC protein, respectively. On the other hand, DR1471 exhibits a high similarity over the whole length of its sequence with putative SMC proteins from other members of the Deinococcaceae family: 71% identity with Dgeo0822 from *D. geothermalis* (Makarova et al. 2007) and 69% identity with Deide08800 from *D. deserti* (de Groot et al. 2009).

SMC protein is dispensable for growth and cell viability

To examine the function of the SMC protein in *D. radiodurans*, we constructed a deletion mutant by replacing the wild-type *smc* gene by a chloramphenicol resistance cassette (Fig. 1). Disruption of the *smc* gene in most bacteria results in severely impaired growth or in a lethal thermosensitive phenotype (Britton et al. 1998; Jensen and Shapiro 1999; Moriya et al. 1998). Surprisingly, the loss of SMC protein in *D. radiodurans* did not affect bacterial growth at 30°C, the optimal growing temperature, as well as at 37°C (Fig. 2). Homogenotes of the Δ*smc* allele were easily obtained after two cycles of purification on selective medium. However, we cannot exclude the possibility that they acquired some compensatory mutation(s) that might relieve a growth defect. Because it has been shown that the growth defects of *smc* mutants in *E. coli* are suppressed by additional mutations in one of the topoisomerase genes (Adachi and Hiraga 2003; Sawitzke and Austin 2000), we sequenced the coding and the promoter regions of the three topoisomerase genes identified in *D. radiodurans* (White et al. 1999): topoisomerase IA (*topA*), DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IB (*topB*). However, no mutations were detected in these genes in the *smc* null mutant.

To further check the dispensability of the *smc* gene in *D. radiodurans*, we used a new diagnostic test discriminating

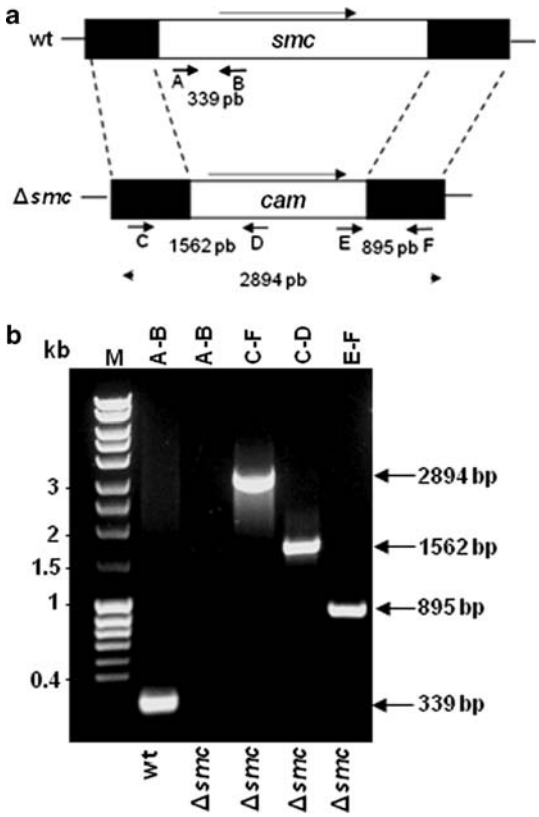

Fig. 1 Schematic representation and test of the deletion–substitution *smc* mutant. **a** Schematic representation of the allele replacement event in the *smc* gene. *Short arrows* indicate the position of primers used for diagnostic PCR. **b** Analysis of a candidate *smc* mutant. The primer pairs used for PCR are indicated on *top* of each *lane*. Expected sizes of the wild type and the mutant fragments are given on the *right* and the size markers (*M*) on the *left*

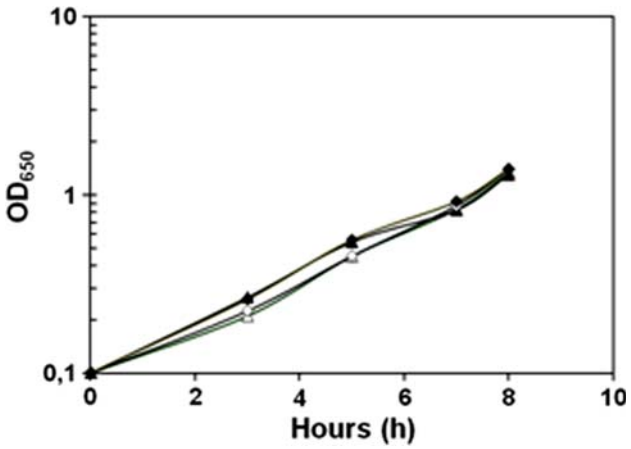

Fig. 2 Effect of a *smc* deletion on the *D. radiodurans* cell growth. Wild type (*diamonds*), Δ*smc* (*triangles*), *open symbols* cultures growing at 30°C, *closed symbols*, cultures growing at 37°C

whether a gene is essential or not (Nguyen et al. 2009). In this system, to avoid the selection of unwanted extragenic suppressors, gene disruption is carried out in diploid cells

containing an additional copy of the disrupted gene on a plasmid (p-*rep*Ts) thermosensitive for its replication. The resulting strain was as resistant to gyrase inhibitors as wild type (data not shown) contrary to the strain devoid of the *smc* gene (see below) proving the functionality of the plasmidic *smc* gene. If the disrupted gene is an essential gene, the cells will lose their viability when shifted to the non-permissive temperature (37°C) that leads to loss of the plasmid. As can be seen in Fig. 3, the Δ*smc* (p-*rep*Ts-*smc*$^{+}$) cells did not show any growth defect at the non-permissive temperature, confirming that the *SMC* protein is not required for cell viability in *D. radiodurans*.

The viability of the Δ*smc* cells raised the possibility that other SMC-like protein(s) might partially compensate for the absence of SMC. The SbcC subunit of the SbcCD complex appeared to be a good candidate because of its structural similarity with the SMC protein (Connelly et al. 1998). To test this hypothesis, we examined whether the depletion of the SbcCD complex in a Δ*smc* background would result in impaired growth. We found that growth of the wild-type strain, the Δ*smc,* and Δ*sbcCD* mutants, and the Δ*smc* Δ*sbcCD* double mutant, were very similar (data not shown).

Loss of SMC and SbcCD proteins renders the cells hypersensitive to DNA gyrase inhibitors

A striking feature of Δ*smc* mutants in *B. subtilis* (Lindow et al. 2002a) and *E. coli* (Adachi and Hiraga 2003; Onogi et al. 2000; Weitao et al. 1999) is their hypersensitivity to gyrase inhibitors. Thus, we tested the sensitivity of the *D. radiodurans smc* null mutant to two gyrase inhibitors, novobiocin and nalidixic acid. For this purpose, the Δ*smc* bacteria were plated on plates containing 20 ng ml^{-1} of novobiocin or 40 μg ml^{-1} of nalidixic acid, drug concentrations that are not lethal for the wild-type strain. As shown in Fig. 4, the Δ*smc* mutant was significantly more sensitive to both drugs than the wild type, because its survival decreased more than tenfold. No such mortality was observed when the mutant cells expressed the *smc* gene *in trans* from a plasmid, indicating that their hypersensitivity to inhibitors was due to the absence of the SMC protein (Fig. 4). The Δ*sbcCD* mutant also showed an increased sensitivity to novobiocin and nalidixic acid (approximately 100-fold decrease in survival) and this phenotype was exacerbated in the Δ*smc* Δ*sbcCD* double mutant (Fig. 4).

To test the effect of SMC protein on the level of DNA supercoiling, we compared the topoisomer distribution of a plasmid isolated from wild type or Δ*smc* hosts. To prepare a reporter plasmid of an appropriate size for this type of analysis, we shortened the shuttle vector p11520 (Bentchikou et al. 2007) giving rise to plasmid p11975 that was stably maintained in Δ*smc* cells as well as in the wild type (data not shown). Purified plasmid DNA was subjected to electrophoresis on agarose gels containing chloroquine (a DNA intercalator) to resolve topoisomers. The topoisomer distributions of the plasmid DNA isolated from the wild type and the Δ*smc* host were identical (Fig. 5). A wild-type pattern of topoisomers was also observed for plasmids extracted from Δ*sbcCD* or Δ*smc* Δ*sbcCD* hosts, indicating that neither SMC nor SbcCD proteins significantly affect

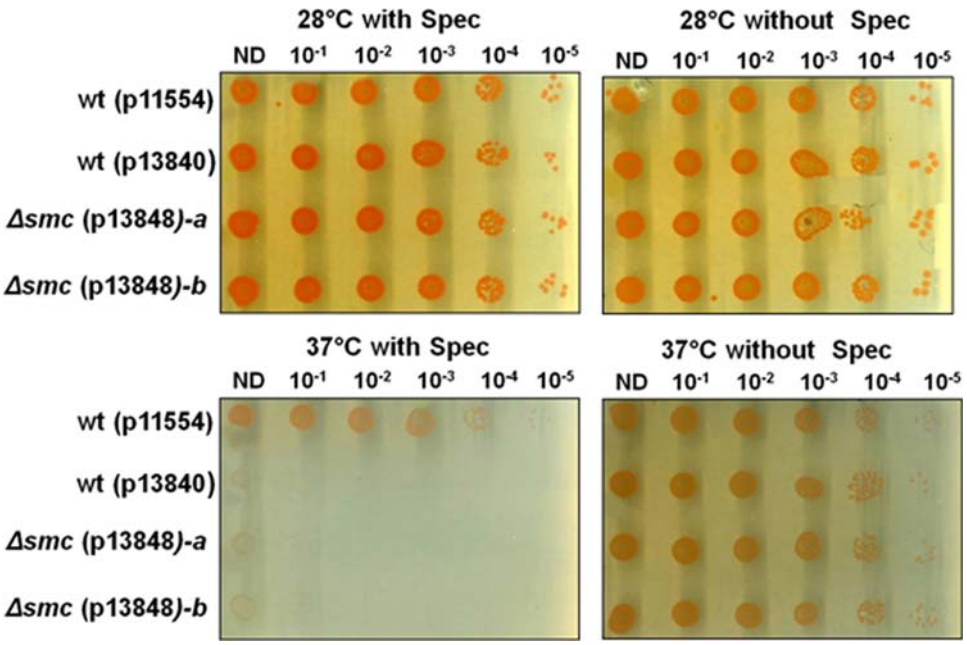

Fig. 3 Test of *smc* gene essentiality using a thermosensitive plasmid. Diluted samples of cells grown at 28°C in liquid medium containing spectinomycin were spotted on plates with or without spectinomycin (*spec*) and incubated at 28 or 37°C as indicated. *Lane 1* [wt (p11554)]: strain GY13785 containing the shuttle vector p11554, *lane 2* [wt (p13840)]: strain GY13781 containing its *repU*$_{Ts}$ derivative p13840, *lanes 3* and *4* [Δ*smc* (p13848)]: two different isolates (*a*, *b*) of strain GY13787 [Δ*smc*/p13848 (*repU*$_{Ts}$ P$_{Spac}$*::smc*)]

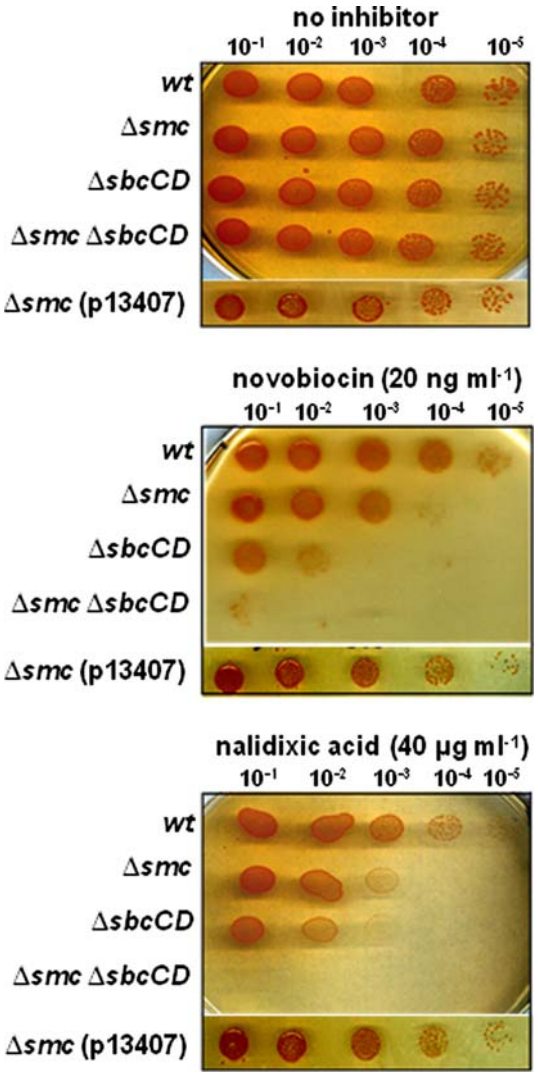

Fig. 4 Survival of wild type, Δ*smc*, Δ*sbcCD*, and Δ*smc* Δ*sbcCD* mutants after treatment with gyrase inhibitors. Serial dilutions of cultures were spotted on plates in the presence or absence of novobiocin or nalidixic acid, as indicated. Test of GY13335: Δ*smc* (p13407) bacteria was performed on plates supplemented with 1 mM IPTG

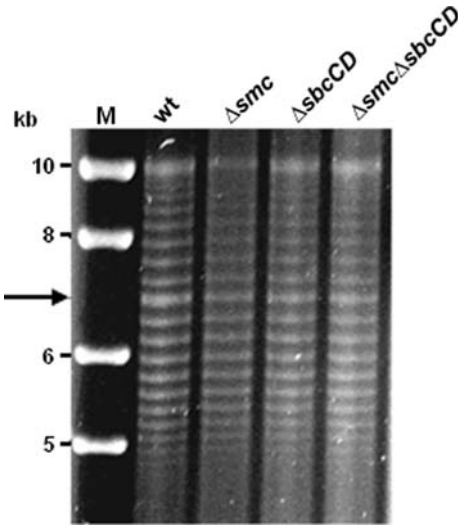

Fig. 5 Supercoiling of plasmid p11975 isolated from wild type, Δ*smc*, Δ*sbcCD* or Δ*smc* Δ*sbcCD* strains. Purified plasmid was subjected to electrophoresis on a 1.8% agarose gel containing 15 μg ml^{-1} chloroquine. The *arrow* indicates the position of linear plasmid DNA

the level of supercoiling of plasmid DNA, although these proteins might affect supercoiling of the chromosome.

Global compaction of the nucleoid and radioresistance are not affected in cells devoid of SMC protein

We analyzed the nucleoid morphologies in *smc* mutant cells by epifluorescence and deconvolution microscopy (Fig. 6). No significant difference was observed between the Δ*smc*, Δ*sbcCD,* Δ*smc*Δ*sbcCD* mutants and the wild-type strain. Likewise, there was no significant increase in the number of anucleate cells (<1%) in the mutant cells, a value close to that observed in the wild type (data not shown). Moreover, cells devoid of SMC protein showed a wild-type radioresistance, as indicated by their survival after γ-ray irradiation (Fig. 7). Similarly, the loss of SMC did not increase the radiosensitivity of the Δ*smc*Δ*sbcCD* double mutant as compared to the single Δ*sbcCD* mutant (Fig. 7) indicating that the SMC protein, in contrast to the SbcCD complex (Bentchikou et al. 2007), is not involved in DNA repair.

SMC protein localizes to discrete foci at the outer edge of the nucleoid

To visualize SMC by immunofluorescence microscopy, we fused a SPA-Tag (Zeghouf et al. 2004) to the C-terminal end of the protein. The SPA-tagged *smc* gene was constructed in vitro (see "Materials and methods") and used to replace the wild-type *smc* allele to yield strain GY12820. The functionality of the tagged protein was tested by showing that it did not confer hypersensitivity to gyrase inhibitors. Because the tagged protein expressed from its natural promoter was barely detectable by Western blotting (Fig. 8a, GY12820), we used a strain (GY13335) expressing the tagged SMC protein from a P_{Spac} promoter in immunofluorescence microscopy experiments. In this strain, the expression of SMC–SPA protein can be induced by IPTG to a detectable level (Fig. 8a). The results of immunofluorescence staining showed that SMC–SPA localized to discrete foci, with one to three brighter foci per cell (Fig. 8b). However, although these foci were in tight contact with the nucleoid, they were located at the outer edges of the nucleoid.

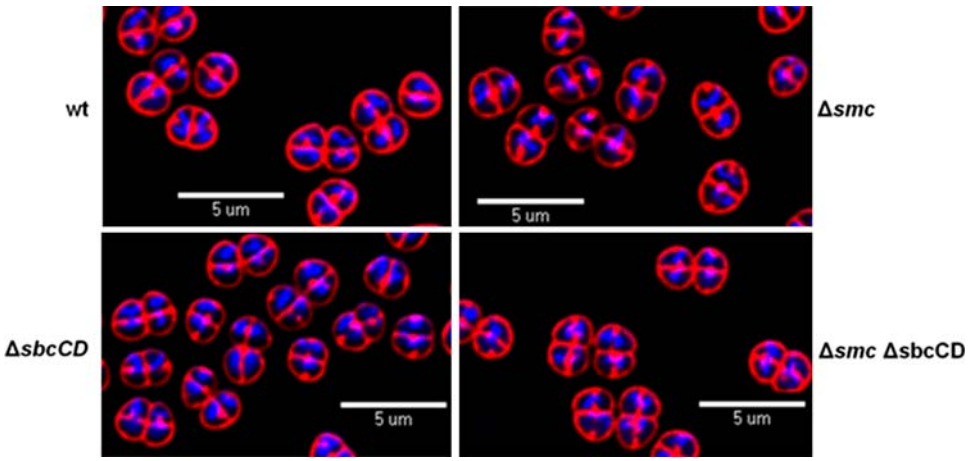

Fig. 6 Cell morphology in the wild type, Δ*smc*, Δ*sbcCD* and Δ*smc* Δ*sbcCD* strains. Nucleoids stained with DAPI appear *blue* and membranes stained with FM4-64 appear *red*. Cells were observed with a wide-field three-dimensional microscope. The images shown are single sections of a deconvoluted Z-series

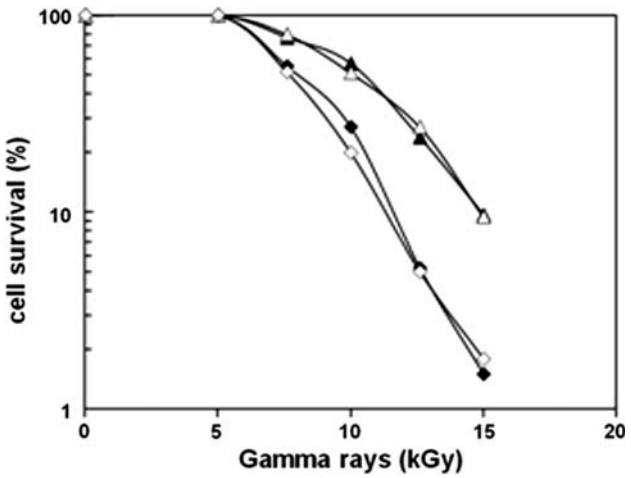

Fig. 7 Survival of wild type, Δ*smc*, Δ*sbcCD* and Δ*smc* Δ*sbcCD* strains after γ-ray irradiation. Wild type (*open triangles*), Δ*smc* (*closed triangles*), Δ*sbCD* (*open diamonds*), Δ*smc*-Δ*sbcD* (*closed diamonds*). Each value is the average of three independent experiments with standard deviations that did not exceed 10% of the mean values

Discussion

In order to gain insight into the highly condensed structure of the *D. radiodurans* nucleoid, we investigated the role of the SMC protein in this organism. The SMC family of proteins exists in virtually all organisms and plays a crucial role in chromosome condensation, pairing, and/or segregation (For reviews, see (Hirano 2005; Strunnikov 2006).

The s*mc* null mutants in *D. radiodurans* showed no growth defect or loss of viability, had normal nucleoid shape and nucleoid segregation, were as radioresistant as the wild type, but were hypersensitive to gyrase inhibitors such as novobiocin and nalidixic acid. In most bacterial species, the loss of SMC or SMC-like proteins results in a more severe phenotype, including reduced growth, thermosensitivity, abnormal nucleoid segregation, and increased sensitivity to gyrase inhibitors (Adachi and Hiraga 2003; Britton et al. 1998; Graumann et al. 1998; Jensen and Shapiro 1999, 2003; Lindow et al. 2002a; Sawitzke and Austin 2000). However, it has been recently shown that an *smc* null *Mycobacterium smegmatis* mutant did not have defects in growth and, contrary to an *smc* null *D. radiodurans* mutant, displayed identical susceptibilities to gyrase inhibitors (Guthlein et al. 2008). The unremarkable phenotype of the deinococcal and mycobacterial *smc* null mutants might be due (1) to some redundancy in the proteins that ensure the organization and compaction of the nucleoid in these bacteria. One of the proteins that could play a major role in nucleoid organization and DNA compaction in *D. radiodurans* may be the HU protein. Indeed, it was recently shown that the deinococcal HU protein localizes all over the nucleoid and is essential for cell viability (Nguyen et al. 2009). Moreover, its progressive depletion generates decondensation of DNA before fractionation of the nucleoid and subsequent cell lysis, reinforcing the major role of HU (Nguyen et al. 2009) (2) the deinococcal SMC protein may perform a function fundamentally different from that of SMCs found in other species. It was for example the case for the deinococcal LexA proteins, LexA1 and LexA2, homologous to the LexA protein of *E. coli*, cleaved by activated RecA protein, but not involved in the control of genes associated with DNA damage tolerance (Bonacossa de Almeida et al. 2002; Narumi et al. 2001; Sheng et al. 2004).

The increased sensitivity of the *smc* mutants to gyrase inhibitors suggests that SMC protein might regulate gyrase activity in *D. radiodurans*. SMC might favor the recruitment of gyrase onto DNA, either by interacting directly with it or by stimulating its interaction with DNA. It might also act in conjunction with gyrase to maintain an optimal level of negative supercoiling in the cell. In the absence of SMC protein, the supercoiling density of the chromosome might be sufficient so that the vital functions might be preserved but the cells become highly sensitive to treatments that further decrease negative supercoiling. However,

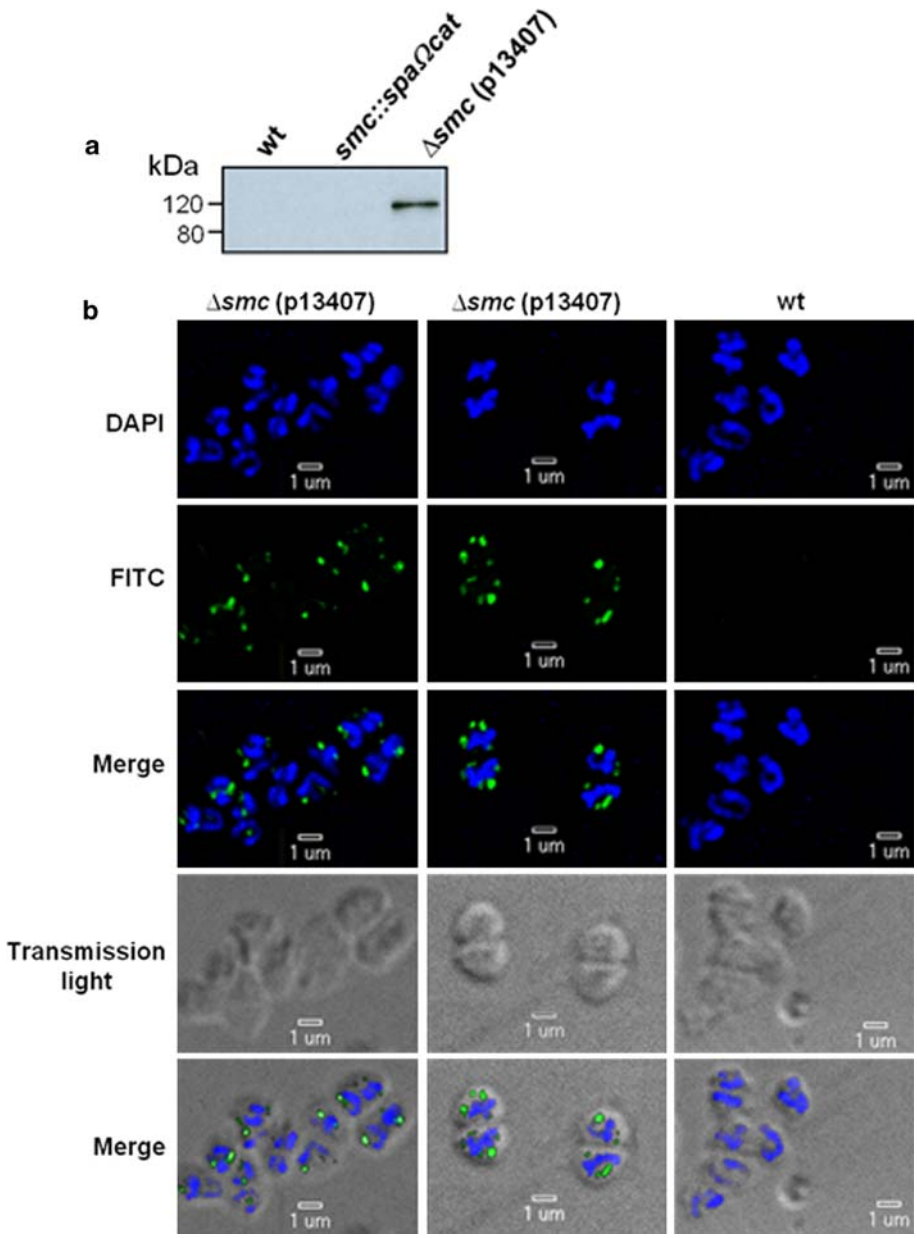

Fig. 8 Localization of SMC–SPA protein in *smc* null cells. **a** Analysis of SMC–SPA protein by Western blot. 7 μg of cell extract from the strains indicated on the top of the immune blot were loaded on SDS-PAGE and the SMC–SPA protein was revealed using an anti-Flag antibody. **b** Cells were probed with an anti-Flag primary antibody followed by an FITC-coupled secondary antibody (*green*) and with DAPI (*blue*). A Nomarski contrast picture of the cells and an overlay of the FITC and DAPI image are shown at the bottom of each series of pictures. As a control, pictures corresponding to wild-type cells subjected to the same treatment are shown. Cells were observed with a wide-field three-dimensional microscope. The images shown are single sections of a deconvoluted Z-series. The *scale bar* 1 μm is applicable to all the cells of a square

we found that the levels of supercoiling of plasmid DNA propagated in the *smc* mutant or in the wild type were indistinguishable. Similarly, the level of plasmid supercoiling was not changed in *E. coli* mutants devoid of the MukB protein, a functional analog of SMC, although there is evidence that MukB is involved in compaction of chromosomal DNA (Sawitzke and Austin 2000). It is possible that plasmid DNA is a poor reporter of the SMC activity if this protein acts locally to assist gyrase in the organization of chromosomal DNA loops.

We observed that the *sbcCD* mutants were also hypersensitive to gyrase inhibitors, and this phenotype was exacerbated in the Δ*smc* Δ*sbcCD* double mutant. The SbcCD complex containing the SMC-like SbcC subunit, is a prokaryotic ortholog of the Rad50/Mre11 eukaryotic complex and was shown to be involved in DSB repair in *D. radiodurans* (Bentchikou et al. 2007). It has been proposed that the SbcCD complex plays a key role in processing of DNA ends especially when they are blocked by covalent binding of proteins (Connelly and Leach 2002; Cromie and Leach 2001; Mascarenhas et al. 2006). This role might explain the hypersensitivity of the *sbcCD* mutant to nalidixic acid, a drug that stabilizes the cleaved gyrase DNA–drug complexes (Sugino et al. 1977). The SbcCD complex, via its nuclease activity, might help the repair of DNA breaks by removing gyrase–drug complexes from the ends. However, this mechanism cannot explain the hypersensitivity of the *sbcCD* mutant to novobiocin, since this drug inhibits gyrase-promoted DNA supercoiling by binding to the ATP site but does not inhibit the DNA

cleavage–religation reaction catalyzed by gyrase (Maxwell 1993). In this case, a more direct role of the SbcCD complex in DNA folding can be proposed. Alternatively, the absence of the SbcCD complex might sensitize the cell to gyrase inhibitors by decreasing the level of supercoiling.

We found that SMC is located in several foci distributed at the periphery of the nucleoid, exhibiting one to three bright foci per cell. The SMC foci are not regularly positioned in each cell, suggesting that the molecules bind randomly to a few distinct DNA regions. Discrete SMC or MukB foci were also observed in *B. subtilis* (Graumann et al. 1998; Volkov et al. 2003), *C. crescentus* (Jensen and Shapiro 2003) and in *E. coli* (Danilova et al. 2007; den Blaauwen et al. 2001), supporting a model in which SMC forms a subcellular structure that locally condenses DNA and organizes the chromosome into structures that facilitate chromosome segregation.

In summary, we found no obvious phenotype linked to the deletion of the *smc* gene in *D. radiodurans*, apart from a hypersensitivity to gyrase inhibitors. Several reasons could explain these results: (1) the cellular amount of SMC in *D. radiodurans* might be too low for the protein to play a major role in DNA condensation (2) SMC acts within complexes with other non-SMC proteins; *D. radiodurans* might lack a subunit essential for the SMC DNA condensing activity. Indeed, in *B. subtilis*, SMC is functional only when associated with an ScpA/ScpB complex (Hirano and Hirano 2004; Mascarenhas et al. 2002; Soppa et al. 2002). In *D. radiodurans*, there is an ScpB homolog, but not an ScpA homolog (White et al. 1999) (3) other proteins might play a major role driving compaction of DNA. We have shown that the SMC-like SbcC protein is probably not involved in this process. A third SMC-like protein, RecN, is also present in *D. radiodurans* cells. It was proposed that RecN acts in DNA repair as a sensor of DSBs (Mascarenhas et al. 2006) and its role in DNA compaction is not known [for review, see (Graumann and Knust 2009)].

A small number of homologs of the classical nucleoid-associated proteins from *E. coli* have been identified in *D. radiodurans*. We speculate that an original set of nucleoid-associated proteins are present in *D. radiodurans* to ensure the strong compaction of the nucleoid that can favor survival when cells are subjected to harmful conditions.

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