The RecBCD and SbcCD DNases suppress homology-facilitated illegitimate recombination during natural transformation of *Acinetobacter baylyi*

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During natural transformation of *Acinetobacter baylyi*, the genomic integration of foreign (non-homologous) DNA is possible when the DNA contains a single segment homologous to the recipient genome (anchor) through homologous recombination in the anchor facilitating illegitimate recombination in the neighbouring foreign DNA (homology-facilitated illegitimate recombination; HFIR). DNA integration by HFIR occurs about 10 000 times less frequently than fully homologous recombination, but at least 100 000-fold more frequently than integration in the absence of any homology. We investigated the influence of the RecBCD enzyme (DNase/helicase) and SbcCD DNase (DNA-structure-specific single-strand endonuclease and exonuclease) on HFIR. In a *recBCD* null mutant the acquisition of foreign DNA was elevated 11-fold relative to wild-type cells by a 6.9-fold increased HFIR frequency and by the integration of longer stretches of foreign DNA in each event. In an *sbcCD* null mutant, the foreign DNA acquisition was 4.5-fold higher than in the wild-type, while homologous transformation with large DNA molecules was unaffected and increased 3.2-fold with small DNA fragments. The *sbcCD* mutation partially suppressed the high UV sensitivity and low viability of the *recBCD* mutant and also decreased its foreign DNA acquisition by HFIR to the lower level of the *sbcCD* mutant. We propose that suppression of HFIR results from the elimination of double-stranded intermediates of the HFIR process during transformation by RecBCD, and by SbcCD interfering with branched molecules. Our results provide evidence that the homologous recombination enzymes RecBCD and SbcCD control the level of foreign DNA acquisition by HFIR.

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INTRODUCTION

In numerous prokaryotes, natural transformation is one of the major horizontal gene transfer processes allowing the intra- and interspecific exchange of genetic information (Lorenz & Wackernagel, 1994; Tønnum *et al.*, 1995; de Vries *et al.*, 2004), and it is a driving force both in the generation of diversity and for evolution (Arber, 2000). During natural transformation, a competent cell binds to a double-stranded DNA molecule, introduces a double-strand break, takes up the DNA, and transfers a single strand into the cytoplasm while the complementary strand is degraded (Dubnau & Provvedi, 2000; Chen & Dubnau, 2004). If the single strand is homologous to the cellular genome, it can be integrated by homologous recombination. The integration of foreign (non-homologous) DNA

during transformation is possible if the foreign DNA is flanked by two segments that provide sequence homology to the recipient genome in which homologous recombination can occur. Foreign DNA having only a single small homologous segment can also be integrated through homology-facilitated illegitimate recombination (HFIR; de Vries & Wackernagel, 2002), which proceeds by homologous recombination in the homologous segment (anchor) accompanied by illegitimate recombination of the adjacent foreign DNA. HFIR was first observed in *Streptococcus pneumoniae* (Claverys *et al.*, 1980), and was subsequently studied in detail in Gram-positive and Gram-negative bacteria, including *S. pneumoniae* (Prudhomme *et al.*, 2002), *Acinetobacter baylyi* (de Vries & Wackernagel, 2002; de Vries *et al.*, 2004; Harms *et al.*, 2007; Hülter & Wackernagel, 2008) and *Pseudomonas stutzeri* (Meier & Wackernagel, 2003, 2005). In *A. baylyi* transformation, HFIR is about 10 000 times less frequent than fully homologous recombination, but at least 100 000-fold more

Abbreviations: Ap, ampicillin; DSB, double-strand break; HFIR, homology-facilitated illegitimate recombination; Km, kanamycin; Km^R, kanamycin resistance.

frequent than integration of foreign DNA by illegitimate recombination in the absence of any homology (de Vries & Wackernagel, 2002; Hülter & Wackernagel, 2008).

Recently it was observed that homologous recombination during transformation of *A. baylyi* is influenced by RecBCD, resulting in fivefold decreased transformation by large chromosomal DNA fragments (≥ 50 kb) and 3.3-fold increased transformation by a 1.5 kb PCR product (Kickstein *et al.*, 2007). In *Escherichia coli*, the RecBCD enzyme is a central recombination enzyme having double-strand-specific DNase and helicase activities, of which the DNase activity is downregulated by specific octanucleotide sequences in DNA termed chi, in which a 3' single-stranded tail is loaded with RecA protein and initiates recombination (Kowalczykowski *et al.*, 1994; Anderson & Kowalczykowski, 1997). The RecBCD enzyme is necessary for the recombinative repair of spontaneous broken replication forks (double-strand end repair) and DNA damage-induced double-strand breaks (DSBs), and for the genomic integration of homologous DNA transferred into the cell by transduction or conjugation (Kowalczykowski *et al.*, 1994; Kuzminov, 1999). To explain the effect of a *recBCD* mutation on transformation of *A. baylyi* (and other transformable bacteria), it has been proposed that the genomic integration of the taken-up single strand occurs frequently by two temporally separated strand invasion and ligation processes for the 5' and 3' ends, which can lead to a DSB, e.g. when a nick is approached in the opposite strand or a replication fork runs through that region, and the DSB in turn will require RecBCD for repair (Kickstein *et al.*, 2007). As in *E. coli*, a *recD* deletion mutant of *A. baylyi* probably retains the helicase activity of RecBCD, which is thought to act together with other DNases such as RecJ to mediate recombination. The *recD* mutant has the same transformation phenotype as the *recBCD*⁺ strain and is not impaired in UV survival and viability (Kickstein *et al.*, 2007). The *recD* mutants of *E. coli* are also recombination proficient, fully viable and not UV sensitive (Amundsen *et al.*, 1986; Kowalczykowski *et al.*, 1994; Churchill *et al.*, 1999; Kuzminov, 1999).

In *E. coli*, the effects of *recB* or *recC* mutations can be suppressed by additional mutations at both the *sbcB* (also termed *xonA*) and the *sbcCD* loci (Kushner *et al.*, 1971, 1972; Lloyd & Buckman, 1985). The *xonA* gene encodes an exonuclease I (ExoI) specific for 3' DNA single strands (Lehman & Nussbaum, 1964). A corresponding gene is absent in *A. baylyi* (Barbe *et al.*, 2004). The *sbcC* and *sbcD* genes in *E. coli* encode the subunits of the heterodimeric SbcCD enzyme, which is a single-stranded DNA endonuclease that cleaves DNA at double-strand/single-strand transitions and in branched structures, including hairpin loops and Y-shaped DNA, and which also has an ATP-dependent exonuclease activity (Connelly & Leach, 1996; Connelly *et al.*, 1997, 1998, 1999). SbcC belongs to the superfamily of SMC (structural maintenance of the chromosome) proteins (Connelly *et al.*, 1998) that operate in DNA repair and in genome maintenance and partition

processes. Orthologues of SbcC and SbcD are present in many organisms from all three kingdoms of life (Sharples & Leach, 1995), including *A. baylyi* (Barbe *et al.*, 2004). In the *E. coli* wild-type background, *sbcC* or *sbcD* mutations have no strong phenotype, whereas in *Deinococcus radiodurans* they cause reduced survival after gamma irradiation (Bentchikou *et al.*, 2007). In *Bacillus subtilis*, the SbcCD complex is involved in DNA repair and is then mostly colocalized with the replication machinery (Mascarenhas *et al.*, 2006).

Since RecBCD affects natural transformation in *A. baylyi*, we asked whether the enzyme also interfered with foreign DNA acquisition by HFIR. We found this to be the case, as HFIR was increased in a *recBCD* mutant. In *A. baylyi* the elimination of the genes similar to *sbcC* and *sbcD* of *E. coli* partially suppressed the UV sensitivity and low viability of a *recBCD* mutant and also lowered the increased HFIR frequency. In a *recBCD*⁺ background the *sbcCD* mutation increased HFIR frequency moderately and elevated homologous transformation with short but not long DNA fragments. How RecBCD and SbcCD interfere with HFIR events is discussed.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *A. baylyi* strains JV28 ('wild-type') and its derivatives KOM18 ($\Delta recBCD$) and EK6 ($\Delta recD$) have been described previously (de Vries *et al.*, 2003; Kickstein *et al.*, 2007). The strain KOM17 ($\Delta sbcCD$) was constructed by allelic exchange as described for the generation of the *recBCD* deletion strain (Kickstein *et al.*, 2007). Briefly, DNA segments upstream (1052 bp) and downstream (992 bp) of the *sbcD*, *sbcC* genes of *A. baylyi* were amplified by PCR using the primer pairs *sbcD*-up-f (gtaaCAAGGCAGACAAGATCGGTTGC; lower case, non-homologous tail) and *sbcD*-up-r (atctagaGCTGATGAATTTCTG-TTTTGTATGAG; underlined, *XbaI* restriction site), and *sbcC*-down-f (cttctagaGCTCAAGTCGAATTGAAGTGGTTCG) and *sbcC*-down-r (aacttGAGCTCAATCTCGCCATAAACCGTTG), respectively, and cloned into the plasmid vector pGT41 upstream and downstream of a gene cassette consisting of a selectable (*nptII*, kanamycin resistance, Km^R) and counterselectable (*sacB*, sucrose sensitivity) marker pair, generating pKH80 with a $\Delta sbcCD::(nptII\ sacB)$ allele embedded into the natural flanking genomic regions. This DNA was used to naturally transform JV28, giving strain KOM12, which has the *sbcCD* operon substituted by the *nptII sacB* cassette. From pKH80, the *nptII* and *sacB* genes were removed by cleavage with *XbaI*, resulting in pKH81 with a $\Delta sbcCD$ allele that was used to transform KOM12, giving strain KOM17 ($\Delta sbcCD$; sucrose resistant, kanamycin sensitive). The double mutant strain KOM45 ($\Delta recBCD\ \Delta sbcCD$) was constructed correspondingly by introduction of the $\Delta recBCD::(nptII\ sacB)$ allele into KOM17 by transformation with pKH83 and subsequent transformation of the resulting strain with pKH84, which contained the $\Delta recBCD$ allele. The presence of deletions in strains was verified by PCR across the deletions and by PCR with internal primers for the deleted gene. Cloning of DNA fragments was carried out by standard procedures (Sambrook *et al.*, 1989) using *E. coli* SF8 *recA* (Romanowski *et al.*, 1993) as host strain. The bacterial strains were grown in LB medium (Sambrook *et al.*, 1989) or in minimal medium, which was M9 (Sambrook *et al.*, 1989) with 20 mM succinate (pH 7.5) as carbon source at 30 °C unless indicated otherwise. If applicable, the media contained the antibiotics ampicillin (Ap; 100 µg

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
A. baylyi BD413 strains		
JV28	<i>trpE27 alkM:: (nptII' tg4) rpoB1</i>	de Vries <i>et al.</i> (2003)
JV28-Km ^R	<i>trpE27 alkM:: (nptII⁺ tg4) rpoB1</i>	de Vries <i>et al.</i> (2003)
KOM18	JV28 Δ <i>recBCD</i>	Kickstein <i>et al.</i> (2007)
EK6	JV28 Δ <i>recD</i>	Kickstein <i>et al.</i> (2007)
KOM12	JV28 Δ <i>sbcCD:: (nptII sacB)</i>	This work
KOM17	JV28 Δ <i>sbcCD</i>	This work
KOM45	JV28 Δ <i>recBCD</i> Δ <i>sbcCD</i>	This work
Plasmids		
pGT41	Derivative of pPCR-Script Cam containing <i>bla cat nptII sacB</i>	Kickstein <i>et al.</i> (2007)
pKH80	pGT41 Δ <i>sbcCD:: (nptII sacB)*</i>	This work
pKH81	pGT41 Δ <i>sbcCD*</i>	This work
pKH83	pGT41 Δ <i>recBCD:: (nptII sacB)*</i>	Kickstein <i>et al.</i> (2007)
pKH84	pGT41 Δ <i>recBCD*</i>	This work
pBlue-Km1	Derivative of pBluescript containing <i>nptII bla</i> (4.8 kb)	de Vries & Wackernagel (2002)
pBlue-Km-tg4	Derivative of pBluescript containing <i>nptII tg4 bla</i> (5.2 kb)	de Vries <i>et al.</i> (2003)

*These plasmids contain upstream and downstream border sequences of the deleted genes indicated (see Methods).

ml⁻¹) or kanamycin (Km; 10 µg ml⁻¹). The presence of the *sacB* gene was screened for by the inability of *sacB*⁺ strains to grow on medium containing sucrose (50 mg ml⁻¹). PCR reactions were carried out with Phusion polymerase (Finnzymes) for cloning steps or for templates larger than 2 kb, or with *Taq* polymerase (Molzym) for other templates, according to the manufacturers' instructions. When Phusion was used, DMSO (10 %) was included in the assays.

Natural transformation of *A. baylyi*. Preparation of competent cultures and transformation assays have been described (de Vries & Wackernagel, 1998). *A. baylyi* was grown in LB broth at 30 °C to 1 × 10⁹ cells ml⁻¹ and frozen in concentrated (1 × 10¹⁰ cells ml⁻¹) aliquots in LB with 20 % glycerol (v/v) at -80 °C until use. For the transformation assay a freshly thawed bacterial suspension was diluted to 2.5 × 10⁸ cells ml⁻¹ in 1–10 ml LB with donor DNA (100 ng ml⁻¹), aerated for 90 min, and plated on M9 or LB medium containing appropriate antibiotics (to examine transformants) and on LB medium (to determine c.f.u.). Colonies were counted after 16–40 h at 30 °C. Transformation frequencies are given as transformants per c.f.u. determined in each experiment. In transformation experiments with linearized plasmid DNA, a few transformants that had formed by cointegration of reconstituted plasmid DNA molecules (resulting from rare non-cleaved plasmid donor DNA) were identified by their Ap resistance (which was screened for subsequently to the selection of transformants on Km medium). These transformants (less than 10 % of the total) were not considered in the calculation of HFIR frequencies and were excluded from the analyses.

Characterization of HFIR transformants. The locations of illegitimate fusion sites in HFIR transformants were determined by PCR amplification of the region using primer F1 (ATTCG-CAGCGCATCGCCTTC) as forward primer and R5 (CTGAAT-GTCGTTGTAAGTGG) or R9 (GATGGCGTACAGCTACTTGG) as reverse primer, and sequencing of the PCR products as reported previously (Harms *et al.*, 2007). Each transformant came from a small separate transformation experiment to ensure that independent transformants were examined.

UV irradiation. Cells were grown in LB exponentially to 2 × 10⁸ cells ml⁻¹ and resuspended in phosphate buffer at the same titre. Aliquots

were exposed to UV light with stirring, as described by Thoms & Wackernagel (1982). The dose rate was 1.8 J m⁻² s⁻¹. Cells were plated after appropriate dilution on LB and counted after incubation for 24 h at 30 °C in the dark.

RESULTS

HFIR is increased in a *recBCD* but not in a *recD* mutant strain

The *A. baylyi* parental strain JV28 and its *recBCD* deletion derivative KOM18 both contain in their genomic *alkM* gene a truncated *nptII* gene that lacks the 3'-terminal 51 bp (*nptII'*; no Km^R) followed by the eukaryotic transcription terminator *tg4* (de Vries *et al.*, 2003). When these strains were transformed by *ScaI*-linearized pBlue-Km1 plasmid DNA containing a complete *nptII* gene (*nptII*⁺) followed by DNA heterologous to *tg4* and the genomic sequence, Km^R transformants could only form by HFIR (Fig. 1a). In the *recBCD* mutant the frequency of HFIR transformants was 6.9-fold higher than in the wild-type (Fig. 1a). The increased HFIR frequency indicated that the RecBCD enzyme suppresses HFIR in the wild-type.

In control transformation experiments with *ScaI*-linearized plasmid pBlue-Km-tg4 DNA, Km^R transformants could form by recombination in two homologous flanks, *nptII'* and *tg4*, which occurred about 10⁴-fold more frequently than HFIR (Fig. 1b). In the *recBCD* strain, the frequency was 3.4-fold higher than in the wild-type, confirming that RecBCD interferes with transformation by short homologous DNA fragments (Kickstein *et al.*, 2007), which were present here as plasmid DNA fragments (5.2 kb). In contrast, when the homologous *nptII*⁺ *tg4* cassette was present in large chromosomal donor DNA fragments [DNA

Fig. 1. Schematic illustrations of HFIR using pBlue-Km1 (a) and homologous recombination using pBlue-Km-tg4 (b) plasmid donor DNA as paradigms (top row). In both cases the recipient (bottom) is the *A. baylyi* chromosome. Sequence homology between donor and recipient DNA is indicated by dotted areas (A, 1110 bp; B, 939 bp). When the donor DNA is JV28-Km^R chromosomal DNA instead of pBlue-Km-tg4 DNA, the areas of homology are not limited on the left of *nptII* and on the right of *tg4*. Homologous recombination events are symbolized by two crossed lines. The illegitimate fusion between donor and recipient DNA is indicated by a single line. *nptII*, Km-resistance gene; *tg4*, eukaryotic transcription terminator. Below the illustrations are tabular listings of HFIR transformation frequencies (a), and of homologous transformation frequencies obtained with pBlue-Km-tg4 plasmid DNA and JV28-Km^R chromosomal donor DNA (b), for the strains indicated in the left-hand column. The data are means $\pm$ SD from *n* independent experiments. *Results significantly different from those with the wild-type by *t* test ($P < 0.01$).

isolated from strain JV28-Km^R carrying a genomic *alkM::(nptII⁺ tg4)* fusion; Table 1], the Km^R transformation frequency of *recBCD* cells was twofold lower than that of wild-type cells (Fig. 1b). In previous experiments the *recBCD* mutation decreased transformation more strongly with chromosomal *trp⁺* DNA (fivefold; Kickstein *et al.*, 2007). We confirmed the stronger effect with genomic DNA fragments carrying a *bla* (Ap-resistance) gene inserted in the *benK* gene giving Ap-resistant transformants fivefold less frequently in *recBCD* [$6.4(\pm 6.0) \times 10^{-4}$] than in wild-type [$3.1(\pm 0.8) \times 10^{-3}$]. Other experiments with chromosomal donor DNAs gave 7.5-fold [ACIAD2185::*nptII*; $4.2(\pm 0.4) \times 10^{-3}$ in wild-type, $6.4(\pm 1.0) \times 10^{-4}$ in *recBCD*] and 10-fold decreases [*ΔrecO::(nptII sacB)*; $4.4(\pm 1.3) \times 10^{-3}$ in wild-type, $4.4(\pm 1.8) \times 10^{-4}$ in *recBCD*]. The reason for the relatively weak effect of *recBCD* deficiency on transformation with the *alkM::(nptII⁺ tg4)* cassette compared to the four other markers is not known.

In a *recD* mutant (strain EK6) the HFIR transformation frequency was identical to that in the wild-type (Fig. 1a) and also homologous transformation was not different (Fig. 1b), suggesting that the RecBC(D⁻) enzyme, perhaps in cooperation with other DNases, acts similarly to the RecBCD enzyme in both HFIR and homologous transformation.

More foreign DNA is integrated per HFIR event in the *recBCD* mutant

Illegitimate fusion sites identified in 22 independent HFIR transformants (each taken from a separate transformation experiment) of the *recBCD* mutant were dispersed over the entire heterologous donor DNA and the corresponding recipient region (Fig. 2a). The sites were somewhat shifted away from the anchor region compared to the fusion-site pattern observed in the wild-type, which involved the same nucleotide sequences in donor and recipient DNA (Fig. 2c; Harms *et al.*, 2007). The mean length of foreign DNA integrated per event in the *recBCD* mutant was 1118 nt, which was significantly higher than the 682 nt integrated in the wild-type (significant at the 5% level; Mann-Whitney U test; $P = 0.032$). The mean length of deleted recipient DNA was 1107 nt, which was not significantly different from the 732 nt in the wild-type ($P = 0.1$). The *recBCD* mutation led to 11-fold higher foreign DNA acquisition compared to wild-type (Table 2). The molecular characteristics of the illegitimate fusion sites in the *recBCD* mutant, including the mean length of microhomologies (8 nt), their GC contents (58%) and the free energy of hybridization [$-17 \text{ kcal mol}^{-1}$ ($-71.13 \text{ kJ mol}^{-1}$); Harms *et al.*, 2007], were not different from those of the sites in the wild-type [8 nt, 57%, and $-14.3 \text{ kcal mol}^{-1}$ ($-59.83 \text{ kJ mol}^{-1}$), respectively; Harms *et al.*, 2007].

Fig. 2. Locations of illegitimate fusion sites in HFIR transformants of KOM18 (a; $\Delta recBCD$; $n=22$), KOM17 (b; $\Delta sbcCD$; $n=22$) and JV28 (c; wild-type; $n=23$), respectively. For each individual transformant the illegitimate fusion between donor (top; pBlue-Km1 linearized with *Scal*) and recipient (bottom; chromosome) is indicated by a line. Identical fusions present in several independent HFIR transformants are marked by thick lines and the boxed number of transformants. Primer-binding sites (F1, R5, R9) are indicated by arrowheads. *nptII*⁺, Km-resistance gene; *nptII*['], 3'-terminally truncated *nptII* conferring no resistance; ColE1 origin, origin of plasmid replication; *bla*['], fragment of Ap-resistance gene; *alkM*['], *alkM*['], fragments of the alkane monooxygenase gene; *tg4*, eukaryotic transcription terminator; ACIAD1432, putative ORF.

HFIR is increased by *sbcCD* deficiency

In *A. baylyi*, the genes *sbcC* and *sbcD* homologous to those of *E. coli* encoding the SbcCD DNase form an operon as in *E. coli* (Barbe *et al.*, 2004). The deduced amino acid sequences of SbcC and SbcD have 28 and 30 % identity overall (44 and 46 % similarity), respectively, with the corresponding proteins of *E. coli*. An *sbcCD* deletion mutant of *A. baylyi* was constructed (KOM17), and with this strain HFIR transformants were obtained at a significantly higher (3.8-fold) frequency than with the wild-type (Fig. 1a), suggesting that about 74 % of HFIR events were eliminated in the wild-type by the SbcCD DNase. The distribution of illegitimate fusion sites in 22 independent transformants of the *sbcCD* mutant (Fig. 2b) did not differ discernibly from that found in the wild-type. On average, 821 nt were integrated per HFIR event in the *sbcCD* mutant, so that foreign DNA acquisition by HFIR was 4.5-fold higher compared to wild-type (Table 2). As with the transformants of the *recBCD* mutant, the fusion-site characteristics of *sbcCD* transformants were similar to those of the wild-type (data not shown).

Transformation with short homologous DNA molecules is increased in the *sbcCD* mutant

In vitro the SbcCD DNase has been shown to cleave branched molecules (Connelly & Leach, 1996; Connelly *et al.*, 1997, 1998, 1999). Such structures could form during transformation after invasion of the donor single strand into the recipient DNA. The Km^R transformation frequency of the *sbcCD* mutant with large chromosomal DNA containing the *alkM*::(*nptII*⁺ *tg4*) cassette from strain JV28-Km^R was about as high as that of the wild-type (Fig. 1b). Similar results were obtained in *trp*⁺ transformation experiments with chromosomal DNA [$2.0(\pm 1.3) \times 10^{-3}$ in *sbcCD* and $2.0(\pm 1.4) \times 10^{-3}$ in wild-type], whereas with the small DNA molecules of the linearized plasmid pBlue-Km-*tg4*, the *sbcCD* strain gave a significantly higher (3.2-fold) Km^R transformation frequency than the wild-type (Fig. 1b). This result was confirmed by the significantly (3.9-fold) higher *trp*⁺ transformation frequency in *sbcCD* [$2.3(\pm 1.2) \times 10^{-3}$] compared to wild-type [$5.9(\pm 1.2) \times 10^{-4}$] with the 1.5 kbp *trp*⁺ PCR product (*t* test; $P < 0.01$). The *sbcCD*

Table 2. Amounts of foreign DNA acquired by HFIR in *recBCD* and *sbcCD* mutant strains of *A. baylyi*

Strain (genotype)	Frequency of HFIR events (A)	Mean number of foreign nucleotides integrated per event (B)	Relative amount of acquired foreign DNA by HFIR ($A/A_0 \times B/B_0$)
JV28 (wild-type)	1.8×10^{-7} (A_0)	682 (B_0)	1
KOM18 ($\Delta recBCD$)	1.3×10^{-6}	1118	11
KOM17 ($\Delta sbcCD$)	6.9×10^{-7}	821	4.5

mutant showed the same generation time during exponential growth as the wild-type (46 min) and had full UV repair capacity (Fig. 3).

SbcCD deficiency partially suppresses phenotypes of the *recBCD* mutant and renders the double mutant cold sensitive for growth

As *A. baylyi* lacks a gene homologous to the *E. coli* *recBCD* suppressor gene *xonA*, we investigated whether the *sbcCD* deletion alone would suppress the effects of the *recBCD* deficiency. A *recBCD sbcCD* double mutant (strain KOM45) had a generation time during exponential growth of 52 min, which was clearly shorter than that of the *recBCD* single mutant strain (70 min) and comparable to that of the wild-type and *sbcCD* strains (both 46 min). The *sbcCD* mutation also partially suppressed the UV sensitivity of the *recBCD* mutant (Fig. 3).

We also observed a suppression of the effects of the *recBCD* mutation on HFIR by the *sbcCD* deletion, as the 6.9-fold higher HFIR frequency of the *recBCD* mutant compared to the wild-type was lowered by the *sbcCD* mutation to a level 3.8-fold higher than that of the wild-type (Fig. 1a). Also, the 3.4-fold increased homologous transformation of the *recBCD* mutant strain with linearized plasmid DNA was reduced by approximately one-half to 1.8, and the twofold lower transformation by chromosomal DNA was brought

back to the wild-type level by the *sbcCD* mutation (Fig. 1b). The wild-type-like homologous recombination frequencies were confirmed by transformation with the 1.5 kb *trp*⁺ PCR product and chromosomal *trp*⁺ DNA (data not shown).

As an unexpected side effect, the *recBCD sbcCD* mutant, which grew normally at 30 °C in both solid and liquid media, was found to be cold sensitive for growth on LB medium as colony formation dropped to about 0.3 % at 20 °C (wild-type: 100 %). The cold sensitivity appeared when a *recBCD* mutation was crossed into an *sbcCD* strain and vice versa. The survivors at 20 °C gave identical c.f.u. values at 30 and 20 °C, indicating that they were cold-resistant mutants.

DISCUSSION

The RecBCD enzyme suppresses foreign DNA acquisition during natural transformation by HFIR in wild-type *A. baylyi* cells, as concluded from the 6.9-fold higher HFIR frequency of the *recBCD* mutant. Considering that RecBCD is an exonuclease specific for double-strand DNA ends, while HFIR is thought to occur by homologous recombination of the taken-up single strand at the anchor region combined with illegitimate joining of the heterologous end to the recipient DNA (de Vries & Wackernagel, 2002), this result was intriguing. The increased HFIR frequency suggests that at least 85 % of potential HFIR events pass through a RecBCD-sensitive DNA intermediate. The increased HFIR frequency resembled the higher homologous transformation frequency of the *recBCD* mutant, with relatively short DNA fragments (PCR products or inserts of plasmids) compared to wild-type (Kickstein *et al.*, 2007). The transformation-inhibiting effect of the functional RecBCD enzyme has been explained by genetic marker degradation when the enzyme resects a duplex DNA end during DSB repair to the next chi site at which recombination can initiate (Kowalczykowski *et al.*, 1994). It is thought that DSBs can occur during transformation when only one end of the transforming DNA strand is ligated to resident DNA and a nick is approached in the opposite strand or replication runs through that region (Kickstein *et al.*, 2007). When the transforming DNA molecules are short, resection would frequently destroy the marker or the heterologous DNA end in the course of HFIR, particularly since heterologous DNA would not be protected by a cognate chi site for the RecBCD enzyme of

Fig. 3. Survival of *A. baylyi* strains after UV irradiation. JV28, wild-type (○); KOM17, $\Delta sbcCD$ (△); KOM18, $\Delta recBCD$ (□); KOM45, $\Delta recBCD \Delta sbcCD$ (◇). Data are means from three to four independent experiments; error bars, 1 SD.

A. baylyi (Kowalczykowski *et al.*, 1994). In *recBCD* mutants duplex DNA ends persist longer (Kowalczykowski *et al.*, 1994). It is proposed that in *A. baylyi* persisting duplex DNA ends can undergo a RecBCD-independent repair, which would act on homologous and, less efficiently, on heterologous ends (Fig. 1). The observation that the integrated foreign DNA segments are 1.6-fold longer in the *recBCD* mutant than in the wild-type is in accord with this model (Table 2).

The above considerations have the implication that the majority of HFIR transformants in the *recBCD* mutant were not formed by direct single-strand integration as previously proposed (de Vries & Wackernagel, 2002) but by repair of duplex DNA ends independently of RecBCD. The repair could be similar to that acting in *E. coli* whereby replication blockage elicits a DSB and the restoration of the replication fork proceeds via a microhomology-dependent deletion formation (Bierne *et al.*, 1997). The pathway termed the single-strand annealing repair pathway was proposed to rely on the annealing of a single-stranded overhang at the broken arm with a microhomology in the non-broken molecule. If the microhomology is located ahead of the primary break site, a new replication fork structure is formed at the expense of a deletion (Bierne *et al.*, 1997). Repair with deletion formation increases 15-fold in cells devoid of the nucleolytic activity of the RecBCD enzyme (*recD*), indicating that the extended persistence of the broken DNA arm increases its chance to enter into the single-strand annealing repair pathway (Bierne *et al.*, 1997). Accordingly, a *recBCD* mutation would increase (the microhomology-dependent) HFIR in *A. baylyi*, as was observed. However, a *recD* deletion strain did not (Fig. 1), suggesting a different situation in *recD* mutants of *E. coli* and *A. baylyi*. Possibly, in *A. baylyi* the action of the RecBC(D)⁻ helicase together with that of other exonucleases removes approximately similar amounts of DNA from duplex DNA ends during attempted recombination repair, in the same way as the RecBCD enzyme, which frequently leads to marker loss. In fact, the phenotype of the *recD* mutant of *A. baylyi* with respect to transformation with short and long homologous DNA as well as HFIR transformation was indistinguishable from that of *recBCD*⁺ cells (Kickstein *et al.*, 2007; Fig. 1). Recently, the microhomology-dependent single-strand annealing mechanism has also been proposed to cause the illegitimate recombination between prophage DNA and host genome during the formation of bio-transducing phage lambda in *E. coli* (Shiraishi *et al.*, 2005).

The increased HFIR transformation and increased transformation with short homologous donor DNA in the *sbcCD* mutant can be related to the absence of single-strand cleavage in branched structures such as those formed during strand invasion, which is normally performed by the SbcCD DNase in the wild-type (Connelly *et al.*, 1998, 1999). Cleavage at the branch would lead to the loss of a potential transformant if the genetic marker is located on the cleaved-off single strand, regardless of whether that part

consists of homologous DNA (as in homologous transformation) or heterologous DNA (as in HFIR transformation). With long homologous donor DNA, the cleaved-off large DNA fragment can engage in a subsequent integration process in which it has a further chance to escape SbcCD. This would explain why the *sbcCD* mutation does not have a strong effect on the transformation with chromosomal DNA (compared to the *sbcCD* mutant the transformation frequency of the wild-type with chromosomal DNA was about 0.7; Fig. 1b).

The *sbcCD* mutation decreased the high HFIR level seen in the *recBCD* strain to the lower level of the *sbcCD* strain, acting like a phenotype suppressor. The decreased HFIR frequency of the double mutant was unexpected. We can speculate that the HFIR transformants that occur in the *sbcCD* strain and the *recBCD sbcCD* strain were not formed principally by the repair of transformation-induced DSB but by the initially proposed single-strand integration process (de Vries & Wackernagel, 2002), and that this process is no longer counteracted by the branch-cleaving activity of SbcCD, thus leading to increased HFIR frequencies. At the same time, the many HFIR transformants observed in the *recBCD* single mutant are possibly not formed in the *recBCD sbcCD* strain, perhaps because SbcCD is required for their generation in the single-strand annealing pathway, e.g. through the processing of the annealed strands at the microhomology into ligatable structures by trimming of the non-annealed single-strand tails. The double mutant was also peculiar in its cold-sensitive phenotype. Cold sensitivity has been observed previously in mutants of *E. coli* lacking the four single-strand DNases ExoI, ExoVII, ExoX and RecJ (Burdett *et al.*, 2001), or ExoI, ExoVII, RecJ and SbcCD (Thoms *et al.*, 2008). Inactivation of one of the mismatch repair (MMR) genes restores growth at low temperature to the *E. coli* quadruple single-strand DNase mutants (Burdett *et al.*, 2001; Thoms *et al.*, 2008). In the *A. baylyi recBCD sbcCD* mutant, the introduction of the *mutS::nptII* allele did not restore growth at 20 °C, suggesting that the cold sensitivity is not related to the unsuccessful processing of intermediates of MMR or to hampered recovery from attempted MMR (Burdett *et al.*, 2001).

Taken together, the results indicate that the activities of both RecBCD and SbcCD suppress foreign DNA acquisition, presumably by degradation of intermediate DNA structures in the HFIR process. A corresponding conclusion was recently drawn when a 20-fold increased HFIR frequency was observed in a *recJ* mutant (Harms *et al.*, 2007). In that mutant increased numbers, specifically of small 5' heterologous DNA fragments, were integrated by HFIR, indicating that in the wild-type the 5' single-strand-specific RecJ exonuclease effectively degrades heterologous whiskers post-synaptically before they undergo illegitimate strand joining. The data presented in this report may also suggest that HFIR transformants arise by different pathways that are differently affected by RecBCD and SbcCD.

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