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The RecJ DNase strongly suppresses genomic integration of short but not long foreign DNA fragments by homology-facilitated illegitimate recombination during transformation of *Acinetobacter baylyi*

Klaus Harms, Verena Schön, Eva Kickstein and Wilfried Wackernagel*
Genetics, Department of Biology and Environmental Sciences, Carl von Ossietzky University Oldenburg, D-26111 Oldenburg, Germany.

Summary

Homology-facilitated illegitimate recombination (HFIR) promotes genomic integration of foreign DNA with a single segment homologous to the recipient genome by homologous recombination in the segment accompanied by illegitimate fusion of the heterologous sequence. During natural transformation of *Acinetobacter baylyi* HFIR occurs at about 0.01% of the frequency of fully homologous recombination. The role of the 5′ single-strand-specific exonuclease RecJ in HFIR was investigated. Deletion of *recJ* increased HFIR frequency about 20-fold compared with wild type while homologous recombination was not affected. Illegitimate fusion sites were predominantly located within 360 nucleotides away from the homology whereas in wild type most fusion sites were distal (500–2500 nucleotides away). RecJ overproduction reduced the HFIR frequency to half compared with wild type, and transformants with short foreign DNA segments were diminished, leading to on average 866 foreign nucleotides integrated per event (682 in wild type, 115 in *recJ*). In *recJ* always the 3′ ends of donor DNA were integrated at the homology whereas in wild type these were 3′ or 5′. RecJ apparently suppresses HFIR by degrading 5′ non-homologous DNA tails at the post-synaptic stage. We propose that the RecJ activity level controls the HFIR frequency during transformation and the amount of foreign DNA integrated per event.

Accepted 1 March, 2007. *For correspondence. E-mail wilfried.wackernagel@uni-oldenburg.de; Tel. (+49) 441 798 3298; Fax (+49) 441 798 5606.

Introduction

Natural transformation is a main horizontal gene transfer process in prokaryotes that allows the intra- and interspecific exchange of genetic information (Lorenz and Wackernagel, 1994; Tønjum *et al*., 1995) and is a driving force for the generation of genetic variation and evolution (Arber, 2000). During transformation, a DNA uptake-competent cell binds free double-stranded DNA, introduces a double-stranded break into the DNA and actively transfers one strand into the cytoplasm in 3′-5′ direction while the complementary strand is degraded (Dubnau and Provvedi, 2000; Chen and Dubnau, 2004). If the DNA has sequence similarity to the DNA of the recipient cell, genomic integration can occur by homologous recombination. Foreign DNA cannot be integrated into the genome unless it is embedded within flanking homologous regions in which two homologous recombination events can occur.

After its initial observation in *Streptococcus pneumoniae* R800 (Claverys *et al*., 1980), a recombination mechanism has been studied in some detail which leads to the integration of foreign DNA when this is linked only at one side to a short segment with sequence homology. This process has been termed homology-facilitated illegitimate recombination (HFIR) and has been shown to occur in Gram-positive and Gram-negative bacteria including *S. pneumoniae* (Prudhomme *et al*., 2002), *Acinetobacter baylyi* (de Vries and Wackernagel, 2002) and *Pseudomonas stutzeri* (Meier and Wackernagel, 2003). The foreign DNA becomes integrated into the genome by the combination of a homologous recombination event of the transforming molecule within the homologous region (anchor) and an illegitimate recombination event outside the homology. In this way stretches of thousands of nucleotides covering complete foreign genes can be acquired by the recipient including DNA from another kingdom (de Vries *et al*., 2004). Often the foreign DNA replaces genomic DNA of equivalent length (Prudhomme *et al*., 2002; de Vries and Wackernagel, 2002; Meier and Wackernagel, 2003). In *A. baylyi*, HFIR is generally about 10 000-fold less frequent than homologous

recombination but at least 100 000-fold more frequent than foreign DNA integration in the absence of any homology (de Vries and Wackernagel, 2002). It is crucial for the HFIR process that only a single fusion of the donor DNA molecule occurs within the anchor, resulting in a single-stranded DNA tail which can engage in illegitimate recombination. The illegitimate fusions in *A. baylyi* occur almost always at small stretches of identical nucleotides between donor and recipient DNA (microhomologies; de Vries and Wackernagel, 2002; de Vries *et al*., 2004). Proteins functioning in the illegitimate recombination process in *A. baylyi* are not known at present, and RecA is not required (J. de Vries and C. Rohde, unpubl. obs.).

Here we have investigated the role of RecJ during HFIR. RecJ is a single-strand-specific exonuclease that degrades DNA with specificity for the 5′ end (Lovett and Kolodner, 1989; 1991) and along with orthologous enzymes from prokaryotes and eukaryotes forms a large family of phosphohydrolases (Aravind and Koonin, 1998). RecJ may degrade the single-stranded DNA introduced into the cytoplasm during transformation and could be involved in the single-stranded processing required for both the homologous and the illegitimate fusion steps. The *recJ* gene from *A. baylyi* has been identified (Barbe *et al*., 2004), and the deduced polypeptide sequence (568 amino acids) has 44% identity to RecJ of *Escherichia coli* and 32% to RecJ of *Thermus thermophilus*, both of which have been experimentally verified as 5′ single-strand-specific exonucleases (Lovett and Kolodner, 1991; Yamagata *et al*., 2001). It was recently shown that RecJ of *A. baylyi* partially complements a *recJ* mutant of *E. coli* and that a *recJ* null mutation in *A. baylyi* does not measurably influence growth rate, homologous recombination during natural transformation, and survival after UV irradiation (Kickstein *et al*., 2007). Similarly, a *recJ* mutation in *E. coli* also results in a moderate phenotype that often cannot be distinguished from the wild type in recombination and UV resistance, presumably due to redundant enzymatic functions and pathways in *E. coli* (Viswanathan and Lovett, 1998). In *E. coli*, RecJ is involved in a number of processes, including the RecFOR pathway of homologous recombination (Kolodner *et al*., 1985; Lovett *et al*., 1988). In this pathway, the RecJ exonuclease is thought to act together with the 3′-5′ helicase RecQ by degrading the unwound 5′ strand and thereby to generate a free 3′ strand that can be loaded with RecA and initiate homologous strand invasion, the initial step in homologous recombination. Also in the RecBCD pathway of homologous recombination in *E. coli* and *Salmonella enterica* serovar Typhimurium RecJ is thought to have pre-synaptic roles like trimming of single-strands from duplex DNA ends as well as post-synaptic functions like degrading the strand complementary to the transferred strand for driving strand exchange by removing a competitor strand for pairing (Miesel and Roth, 1996; Razavy *et al*., 1996; Friedman-Ohana and Cohen, 1998; Viswanathan and Lovett, 1998). Furthermore, the RecJ nuclease is necessary for methyl-directed mismatch repair in *E. coli* along with three further single-strand-specific exonucleases providing redundant functions (Burdett *et al*., 2001). These other enzymes are exonuclease I (3′-specific), exonuclease VII (3′- and 5′-specific), and exonuclease X (3′-specific) of which BLAST searches identified an possible orthologue only for exonuclease X in *A. baylyi* (Barbe *et al*., 2004; K. Harms and W. Wackernagel, unpublished). RecJ is also involved in base excision repair (Dianov and Lindahl, 1994). In *E. coli*, *recJ* has a role in illegitimate recombination during UV-induced formation of Lambda *bio*-transducing phages (Ukita and Ikeda, 1996). We have examined the influence of the level of RecJ on the frequency of HFIR events and the amount of integrated foreign DNA per HFIR event by studying wild type and strains with a *recJ* deletion or *recJ*$^+$ overexpression from a multicopy plasmid. The results suggest that RecJ controls the frequency of foreign DNA integrations by HFIR and the amount of foreign DNA integrated per event during natural transformation.

Results

Homology-facilitated illegitimate recombination frequency is increased in a recJ *null mutant*

Homology-facilitated illegitimate recombination events in the chromosome of *A. baylyi* can be detected and quantified by natural transformation of strain JV28 (de Vries *et al*., 2003) which carries a-3′-terminally truncated (51 bp deleted) *nptII* gene in the chromosome conferring no antibiotic resistance, followed by the eukaryotic transcription terminator tg4. If this strain is transformed by DNA containing a complete *nptII* gene but lacking the tg4 transcription terminator region as a second homology, kanamycin-resistant transformants can only form by HFIR (Fig. 1A). When cells of strain JV28 were treated with ScaI-linearized pBlue-Km1 (Fig. 1B) DNA, kanamycin-resistant transformants were obtained at a frequency of $1.8\ (\pm\ 0.9) \times 10^{-7}$. In contrast, when the cells were treated with ScaI-linearized pBlue-Km-tg4 DNA which carries 226 bp foreign DNA between *nptII* and the tg4 transcription terminator (including the 51 bp missing from the 3′ terminus of the recipient *nptII′*) and thus provides two homologous recombination regions of 1110 bp and 939 bp, respectively (Fig. 1A), the transformation frequency was approximately 10 000-fold higher than that of HFIR (Table 1). These findings obtained with chromosomal recombination are similar to the previous measurements of HFIR and homologous transformation which

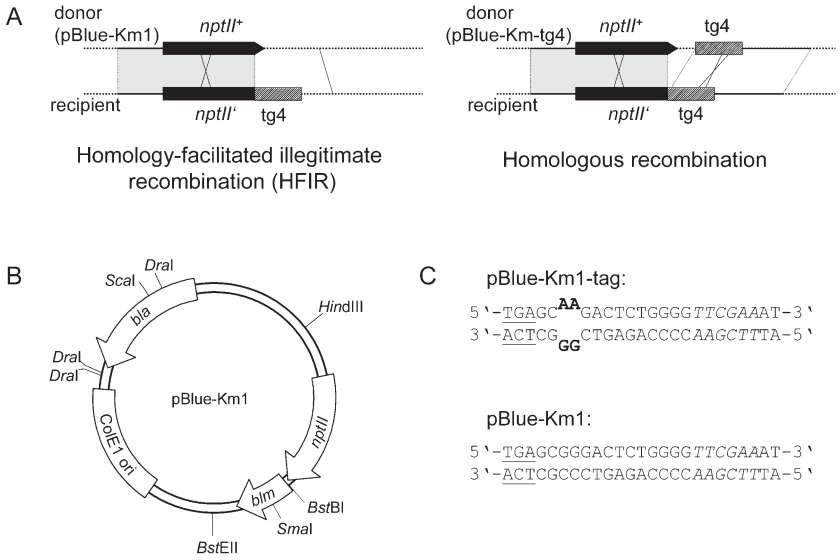

Fig. 1. A. Schematic illustrations of HFIR using pBlue-Km1 (left) and homologous recombination using pBlue-Km-tg4 DNA for transformation (right). The recipient in both cases is the JV28 genome. Regions of sequence homology between the donor (top) and recipient (bottom) are indicated by shaded areas. Homologous recombination events are symbolized by two crossed lines, the illegitimate fusion between donor and recipient is indicated by a single line. *nptII*, kanamycin resistance gene; tg4, eukaryotic transcriptional terminator.
B. Circular map of pBlue-Km1 (4792 bp) with relevant restriction sites. *bla*, ampicillin resistance gene; *blm*, bleomycin resistance gene; ColE1 ori, ColE1 origin of replication. In pBlue-Km-tg4 the region downstream of *nptII*$^+$ is replaced by a region of 939 nucleotides covering tg4.
C. Top: DNA sequence of the molecular tag region from pBlue-Km1-tag. The mismatched base pairs are indicated in bold type. The 3′-terminal nucleotides of *nptII* are underlined. The recognition site for BstBI is marked in italics. Bottom: The corresponding sequence in pBlue-Km1.

were made with partially the same interacting nucleotide sequences located on a plasmid (de Vries and Wackernagel, 2002).

The *recJ* null mutant strain EK4 gave a 20-fold higher HFIR transformation frequency with ScaI-linearized pBlue-Km1 DNA than JV28 (Table 1; statistically significant; $P < 0.01$). However, when EK4 was treated with ScaI-linearized pBlue-Km-tg4 DNA, the transformation frequency was not different from that of the wild type (Table 1). This finding agrees with the unchanged homologous transformation of the *recJ* mutant with chromosomal *trp*$^+$ DNA and with the only slightly elevated transformation frequency by a 1.5 kb polymerase chain reaction (PCR) product covering *trp*$^+$ (Kickstein *et al.*, 2007). These results show that *recJ* deficiency specifically leads to increased HFIR and does not influence homologous recombination.

The illegitimate fusion site distribution is shifted towards short fragment integration in the recJ *strain*

The location of illegitimate fusion sites in HFIR transformants was determined by DNA sequencing. The distribution of the fusion sites of 23 wild type transformants is shown in Fig. 2A. The sites were scattered over the entire segment of heterology of 2569 bp. The shortest distance to the 3′ terminus of *nptII*$^+$ was 14 bp, the longest 2489 bp (29 nucleotides before the end of the

Table 1. HFIR and homologous transformation frequencies and distribution of proximal and distal fusion sites in HFIR transformants.

	HFIR			
Strain	Transformation frequency[a]	Proportion of transformants with proximal[b] illegitimate fusion points (frequency)	Proportion of transformants with distal[b] illegitimate fusion points (frequency)	Homologous recombination transformation frequency[c]
JV28	$1.8 (\pm 0.9) \times 10^{-7}$	30% (5.5×10^{-8})	70% (1.3×10^{-7})	$2.8 (\pm 0.8) \times 10^{-3}$
EK4	$3.7 (\pm 0.9) \times 10^{-6}$	97% (3.7×10^{-6})	2.6% (9.7×10^{-8})	$3.1 (\pm 1.6) \times 10^{-3}$
JV28 pEKX2	$9.5 (\pm 4.1) \times 10^{-8}$	4.5% (4.3×10^{-9})	95% (9.1×10^{-8})	$3.1 (\pm 1.5) \times 10^{-3}$

a. Data are means with standard deviations from five to seven transformation experiments with ScaI-digested pBlue-Km1 DNA.
b. Proximal: up to 308 bp after *nptII*$^+$; distal: 309 bp or more after *nptII*$^+$; the data were from sequencing (see Fig. 2) or by separate PCR-based determination (see *Experimental procedures*).
c. Data are means with standard deviations from three to 14 transformation experiments with ScaI-digested pBlue-Km-tg4 DNA.

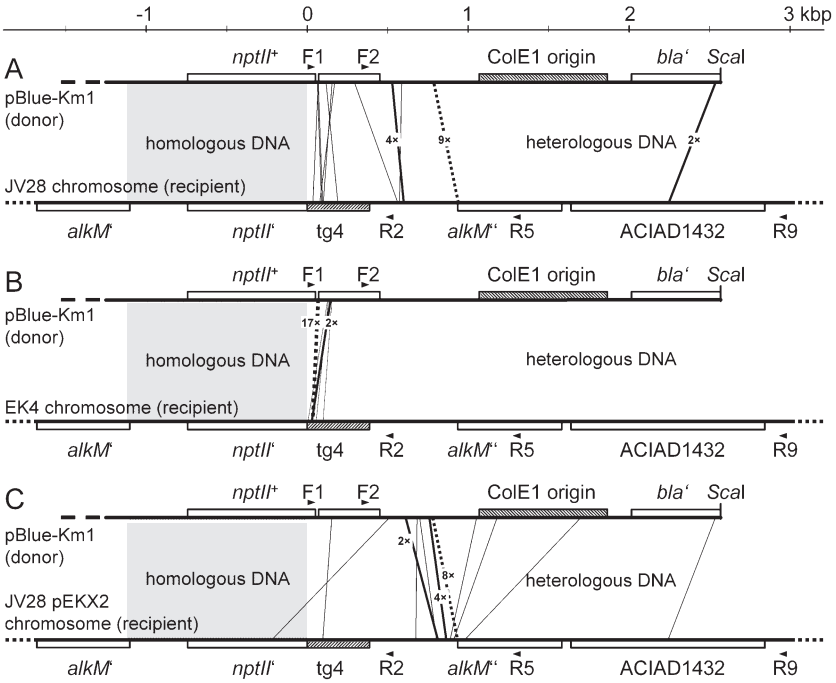

Fig. 2. Locations of illegitimate fusion sites in HFIR transformants of JV28 (A; $n = 23$), EK4 (B; $n = 22$) and JV28 pEKX2 (C; $n = 22$) respectively. For each transformant the fusion between donor (top; ScaI-linearized pBlue-Km1; see Fig. 1B) and recipient (bottom; chromosome) is indicated by a line; multiply used fusion sites are indicated by thick lines and the number of transformants. Primer binding sites are indicated by arrowheads. The hot spots 1 (A and C) and 2 (B) are marked by dotted lines. *alkM'*, *alkM''*: fragments of the alkane monooxygenase gene; ACIAD1423: putative open reading frame.

transforming DNA molecule). The distribution was not even. 30% of the illegitimate fusion sites were located within the first 300 nucleotides after *nptII*$^+$ of the donor DNA. Of the remaining sites, the majority (39% of all isolates) used an identical fusion site (hot spot 1; Fig. 3). Some characteristics of the fusion sites are described in a later section. Among the 23 transformants, the mean length of integrated foreign DNA was 682 nucleotides and the mean length of deleted genomic DNA was 732 bp, leading to an average net loss of 50 bp per HFIR event in the wild type (maximum net gain: 296 bp, maximum net loss: 264 bp).

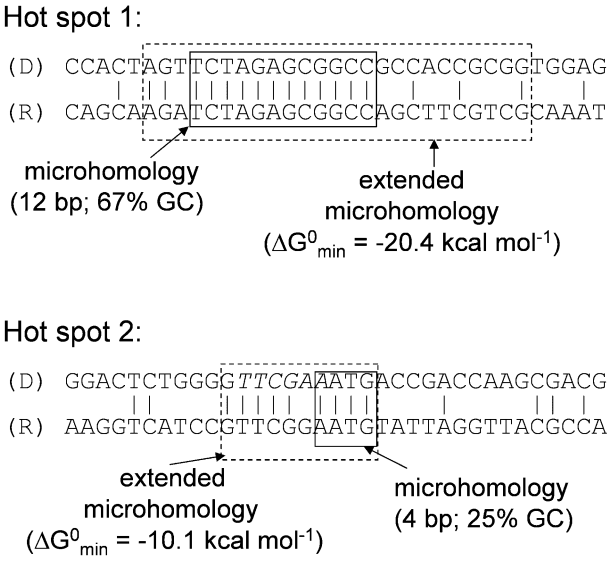

Fig. 3. Nucleotide sequences of illegitimate fusion sites between donor (D) and recipient (R) strands. Microhomologies are indicated by boxes of straight lines, within which the illegitimate fusions had occurred. Extended microhomologies are indicated by boxes of interrupted lines. The ΔG^0_{min} values are calculated for the 3′-homologous donor strand. Top: hot spot 1. Bottom: hot spot 2. For hot spot 2, the overlapping BstBI site is indicated in italics.

The fusion sites of 22 randomly chosen transformants of the *recJ* strain were markedly differently distributed from those of the parental strain (Fig. 2B). All fusion sites were located close to *nptII*$^+$, i.e. within 120 bp after the end (stop codon) of the gene. Of these sites, 17 (77%) were at an identical location 20 bp downstream of *nptII*$^+$ (hot spot 2; Fig. 3). This fusion site was also identified in one of the 23 wild-type transformants. The mean foreign DNA length integrated was 85 bp (significantly different from the wild type as determined by Spearman's rank correlation: $P < 0.01$), and the mean deletion 31 bp (significant; $P < 0.01$). All *recJ* HFIR transformants had a net DNA gain (maximum: 128 bp; minimum: 41 bp). The mean net gain of 54 bp in EK4 was significantly different from the 50 bp net loss in JV28 ($P < 0.01$).

Next, we asked whether the shift of fusion sites in *recJ* towards the anchor resulted from a general shift of all fusion sites or was the consequence of the elimination of fusion events close to the anchor in the wild type. This question was addressed by examining a greater number of transformants for the location of their fusion sites. We screened independent transformants by PCR (see *Experimental procedures*) to determine whether they had proximal fusion sites of the donor DNA (within 308 nucleotides away from the end of *nptII*$^+$) or distal (309 nucle-

otides to the end of the donor DNA). The frequencies of proximal and distal fusion events for JV28 and EK4 transformants are listed in Table 1. We found six distal events among 230 examined transformants of EK4, giving a frequency of about 1×10^{-7} which was similar to the frequency in the wild type. However, the frequency of transformants with proximal fusion sites was about 70-fold higher in *recJ* than in the wild type (Table 1). This result indicates that the overall HFIR frequency increase in *recJ* is due to the appearance of proximal events which are eliminated in the wild type, while the formation of distal fusions is rather unchanged in *recJ*.

RecJ deficiency leads to increased integration of 3′-homologous DNA strands

Previously it was shown that during HFIR in *A. baylyi* transforming single-strands can integrate in the anchor region with the 3′ end or the 5′ end, leaving the 5′ end or the 3′ end for the illegitimate fusion (de Vries and Wackernagel, 2002). It was concluded that homologous 3′ and 5′ single-strands can perform homologous strand invasion. In order to determine the polarity of strand integration within the anchor sequence in the *recJ* mutant, we treated the cells with pBlue-Km1-tag heteroduplex donor DNA and examined the HFIR transformants by tag-specific PCR for integration of the 3′ strand or the 5′ strand (see *Experimental procedures*). The pBlue-Km1-tag DNA was identical in sequence with pBlue-Km1 except for the molecular tags consisting of two mismatched base pairs (AA in the 5′ strand, GG in the 3′ strand) located three bp downstream of the 3′-terminal nucleotide of *nptII* (Fig. 1C). The kanamycin-resistant transformants obtained with pBlue-Km1-tag were clearly distinguished by the PCR. The EK4 transformants (Table 2) all had 3′-homologous and 5′-illegitimate recombination events. We tested for the location of the fusions by PCR analysis and found that in all EK4 transformants the fusion site was proximal (Table 2).

As a comparison we also examined the strand polarity of DNA integration in wild-type HFIR transformants and the proximal and distal location of the illegitimate fusion sites (Table 2). Among transformants with 3′-homologous and 5′-illegitimate recombination events most had proximal sites. Of the transformants with a 5′-homologous integration 66% had their illegitimate fusion sites at a distal position. The data of Table 2 confirm previous results showing 5′ or 3′ fusions in wild-type HFIR transformants (de Vries and Wackernagel, 2002). In the current experiments we observed a larger proportion of 3′-homologous integrations (about 70%) than in the previous experiments (about 50%; de Vries and Wackernagel, 2002) which may be due to the partially different interacting heterologous nucleotide sequences in the two experiments. Perhaps

Table 2. The frequencies of strands with 3′ and 5′ polarity integrated in the homologous region in HFIR transformants and their fusion site position, the HFIR frequencies and the amount of foreign DNA acquired during HFIR in strains with different RecJ levels.

Strain (*recJ*$^+$ expression)	Polarity of integrated homologous strand	Total number	Location of fusion site		Relative frequency of HFIR events (A)	Average acquired nucleotides per event (B)	Relative total acquired nucleotides (A × B)
			Proximal	Distal			
EK4 (deficient)	3′/5′	55/0	55/0	0/0	20	115[a]	2300
JV28 (wild type)	3′/5′	82/29	76/10	6/19	1[b]	682	682
JV28 pEKX2 (overexpression)	3′/5′	18/17	13/8	5/9	0.52	866	450

a. This number was calculated from the wild-type frequency of events with on average 682 nucleotides integrated per event plus the 20-fold higher frequency of events integrating on average 85 nucleotides.
b. The HFIR frequency was 1.8×10^{-7} (see Table 1).

also the RSF1010-based recipient location of the HFIR events in the earlier study may have had an influence although it did not affect the overall frequency.

As the frequency of transformants in *recJ* was 20-fold higher than in the wild type it appears that this increase is due to increased HFIR transformants with 3′-homologous strand usages and proximal fusions. In the wild type, RecJ apparently strongly decreased the number of 3′-homologous strand integrations with proximal fusions. Still these transformants remained the larger group among HFIR transformants (76 of 111; Table 2).

Of the sequenced wild-type HFIR transformants obtained with pBlue-Km1, 30% had proximal and 70% distal fusions (Fig. 2A) whereas among the HFIR transformants obtained with pBlue-Km1-tag heteroduplex DNA more were proximal than distal (Table 2). A reason for the difference is not clear, but it could result from the different DNA preparation procedures (for pBlue-Km1: standard plasmid isolation protocol; for pBlue-Km1-tag: plasmid isolation, restriction, ligation, restriction and gel purification). If single-stranded breaks accumulated in the pBlue-Km1-tag DNA preparation, then the mean size of single-stranded fragments taken up during transformation would decrease and this would reduce the length of the integrated segments.

Overproduction of RecJ decreases HFIR and preferentially reduces the frequency of transformants with short foreign DNA fragment integration

In order to study the influence of elevated RecJ levels on homologous transformation and transformation by HFIR, we employed a strain overproducing RecJ by expression of an IPTG-inducible *recJ*$^+$ gene present on a plasmid (JV28 pEKX2). With this strain grown in the presence of IPTG the homologous transformation frequency was not different from that of JV28 (containing only the vector) when linearized pBlue-Km-tg4 (Fig. 1A) was the donor DNA (Table 1). In contrast, the HFIR transformation frequency of JV28 pEKX2 with linearized pBlue-Km1 DNA was reduced to about half (Table 1).

We determined the location of fusion sites in 22 HFIR transformants of JV28 pEKX2 by sequencing (Fig. 2C). The proportion of proximal fusion sites dropped to 4.5% (1 out of 22; Table 1). The hot spot 1 observed in JV28 was also prominent in JV28 pEKX2 (36% of all fusion sites). The mean length of integrated foreign DNA in the RecJ-overproducing strain was 866 bp (not significantly different from the 682 bp in the wild type as calculated by Spearman's rank correlation; $P = 0.095$) and the mean deletion of chromosomal DNA was 854 bp (not significantly different from the 732 bp in the wild type; $P = 0.28$), leading to an average net DNA gain of 12 bp that was indistinguishable from the average 50 bp DNA loss in the parental strain ($P = 0.86$). The maximum net gain observed in JV28 pEKX2 was 722 bp, the maximum net loss 192 bp. Altogether, RecJ overproduction led to a decrease of the HFIR transformation frequency accompanied by less fusion events proximal to the anchor region (Table 1). While the frequency of transformants with distal illegitimate fusion sites was not influenced by the level of RecJ in the cells (about 1×10^{-7}; Table 1), the frequency of transformants with proximal fusion sites decreased over three orders of magnitude from *recJ* over *recJ*$^+$ to the RecJ-overproducing strain (Table 1).

Compared with wild type the RecJ overproduction in JV28 pEKX2 increased the proportion of 5′-homologous integrations (17 of 35) and also led to more transformants with distal illegitimate fusion points (14 of 35; Table 2). The combination of the relative frequencies of HFIR events and the average foreign DNA acquisition per HFIR event in cells lacking RecJ, with normal RecJ level, and with RecJ overproduction (Table 2) shows that an about fivefold increase of foreign DNA acquisition is associated with the decreasing RecJ level.

The length of foreign DNA to the side of the homology plays a minor role for the formation of HFIR transformants

We examined the influence of the length of heterologous donor DNA downstream of the *nptII*$^+$ of the donor on the frequency of HFIR transformants. Various restriction endonucleases cutting pBlue-Km1 DNA at different positions (Fig. 1B) were used to linearize the plasmid, giving heterologous DNA lengths from 3655 nucleotides down to 18 nucleotides downstream of *nptII*$^+$ (Table 3). The transformation frequencies obtained with these donor DNA preparations are listed in Table 3. In the wild type the frequencies varied somewhat but did not indicate a dependence on the length of the heterologous overhang. Only the BstBI-cleaved DNA with just 18 nucleotides after *nptII*$^+$ gave a very low frequency which may be due to only few nucleotides available for the illegitimate recombination event. In the *recJ* strain all DNA fragments gave significantly higher transformation frequencies, with increases between 4.5-fold and 61-fold. That the BstBI-treated DNA gave transformants at all in the wild type and *recJ* can be explained by the presence of the illegitimate recombination hot spot 2 extending over the nucleotides number 14–23 downstream of the stop codon of *nptII*$^+$ (and the corresponding region in the recipient DNA; Fig. 3). Only in the absence of any exonucleolytic shortening of the transforming DNA occurring on this end, the hot spot 2 can be used for illegitimate recombination which apparently was frequently the case in the *recJ* mutant.

Table 3. HFIR frequencies obtained by transformation of *A. baylyi* JV28 with different restriction fragments of pBlue-Km1.

pBlue-Km1 donor DNA digested with	Number of nucleotides downstream of *nptII*[+a]	Transformation frequency ($\times 10^{-8}$)[b]		
		JV28 (A)	EK4 (B)	(B/A)
HindIII	3651/3655	6.7 ± 1.5	103 ± 34	15
ScaI	2518	18 ± 8.8	372 ± 91	20
DraI	1904	12 ± 3.8	350 ± 69	30
BstEII	569/573	51 ± 22	1030 ± 90	20
SmaI	173	57 ± 21	260 ± 42	4.5
BstBI	16/18	1.3 ± 1.1	78 ± 3	61

a. See Fig. 1B for location of restriction sites on pBlue-Km1. Two numbers indicate the length of the 3′ strand/complementary strand for enzymes producing protruding ends.
b. Data are means with standard deviations of three to seven independent experiments after transformation with digested pBlue-Km1 DNA; the frequencies in EK4 with each DNA preparation were significantly higher than in JV28 (*t*-test; $P < 0.01$).

RecJ does not influence the use of illegitimate fusion sites

The increased HFIR frequency in *recJ* could be caused by RecJ influencing the illegitimate fusion process to use specific fusion sites. The sequenced illegitimate fusion sites (Fig. 2) all contained microhomologies which were preferentially located in GC-rich regions or at GC-rich spots within low GC-regions of both the donor and the recipient strands (data not shown). This is in accord with previous findings involving partially (de Vries and Wackernagel, 2002) or entirely different nucleotide sequences than those employed here (de Vries *et al*., 2004). The mean length of the microhomologies in wild type (8 nucleotides) and RecJ-overproducing cells (7.7) differed from the *recJ* strain (4.4) and also their GC content (wild type: 57%; RecJ-overexpressing strain: 66%; *recJ*: 29%). Although the differences were significant (by Spearman's rank correlation; not shown) it is possible that these differences result from the only limited area in which illegitimate fusions could occur in the *recJ* mutant compared with the *recJ*$^+$ strains (Fig. 2).

The microhomologies at the illegitimate fusion sites were themselves often found within areas of further matching nucleotides interrupted by non-matching nucleotides (termed extended microhomologies; de Vries *et al*., 2004). It is possible that the free energy for hybridization determines the attractivity between donor and recipient nucleotide sequences at an illegitimate fusion site and that RecJ could direct DNA to certain sites. We calculated the minimum free energy (ΔG^0_{min}) for the minimal and extended microhomologies using the nearest neighbour method and base pair parameters given by Wetmur (1996). Two examples are given in Fig. 3. The mean ΔG^0_{min} for all sequenced sites was –14.3 kcal mol^{-1} in the wild type, –15.7 kcal mol^{-1} in the RecJ-overproducing strain, and –10.6 kcal mol^{-1} in *recJ* (differences not significant by Spearman's rank correlation). Thus, the similarity of characteristics of the illegitimate fusion sites suggests that RecJ does not influence the choice of the sites where fusions occur.

Discussion

The *recJ* gene is the first gene identified to influence specifically and strongly HFIR. Its influence was identified by studying the phenotype of a Δ*recJ* mutant of *A. baylyi*. In the *recJ* mutant the HFIR frequencies with donor DNAs having different lengths of heterologous DNA segments to the sides of the homologous anchor sequence were always significantly increased (from 4.5- to 61-fold) compared with the wild type. In contrast, increased homologous transformation in the *recJ* mutant was not observed. The higher HFIR frequencies rested mostly on the higher numbers of transformants with illegitimate fusion sites proximal to the anchor, while the number of transformants with distal fusion sites was almost not influenced. Corresponding with these findings, in a RecJ-overproducing strain the HFIR frequency was decreased compared with the wild type, and this resulted mainly from a decreased number of transformants with proximal fusions. Considering that RecJ is a single-strand-specific 5′ exonuclease, these observations are compatible with the idea that in the wild type RecJ degrades at least portions of the transforming DNA molecules so that molecules with a 5′-ending heterologous part would have a lower chance to form an HFIR transformant. Similarly, molecules having the 5′ end at the anchor part could lose DNA necessary for homologous recombination by degradation which would decrease their chance to engage in HFIR.

The question arises whether degradation of transforming DNA occurs before homologous strand transfer at the anchor (i.e. in the pre-synaptic stage) or after (post-synaptic). A tentative answer can be derived from the absence of an increase of homologous transformation of the *recJ* mutant by relatively short DNA fragments as

reported here (Table 1) as well as by large chromosomal DNA fragments (Kickstein *et al*., 2007). These observations suggest that RecJ does not substantially degrade transforming DNA molecules while present freely in the cytoplasm. This conclusion is supported by the notion that also overexpression of *recJ* did not diminish homologous transformation (Table 1). There was a hint that transformation by shorter homologous DNA fragments than those in Table 1 was slightly increased in *recJ* (Kickstein *et al*., 2007). This point will be discussed below.

A more direct and quantitative answer to the question of pre- or post-synaptic DNA degradation is provided by the experiments identifying the polarity of the integrated homologous sequence during HFIR. Previously it was found that in HFIR transformants of wild type either the 5′- or the 3′-homologous end was integrated (de Vries and Wackernagel, 2002). This finding was confirmed by the experiments reported here and extended to the observation that wild-type transformants with long foreign DNA segments inserted were formed mainly by integration of 5′-homologous strands, while those with short foreign DNA segments inserted (proximal fusions) occurred predominantly by integration of 3′-homologous strands. In contrast, in the *recJ* strain a vast majority of 3′-homologous strand integrations was found. This observation indicates that the strong increase of the HFIR transformant frequency in *recJ* is due to an increase of 3′-homologous integrated strands with 5′ heterologous fusions. These data led to two conclusions about the HFIR process in the wild type. First, 3′-homologous integrated strands are relatively instable at their heterologous 5′ end due to their susceptibility to the RecJ DNase. Thus, the heterologous parts are often fully or partly degraded, leading to a low frequency of HFIR transformants, and the transformants which appear have less often short heterologous segments inserted. This was found (Fig. 2; Table 1), and identified RecJ as a suppressor of HFIR events. Second, 5′-homologous integrated strands are refractory to RecJ at their heterologous 3′ end and therefore these have an increased chance to undergo illegitimate fusion. The relative stability of 3′ tails would result in the wild type in an enrichment of HFIR events with a 5′-homologous integration with short or long 5′-heterologous segments integrated. This was also found (Fig. 2, Table 1). An inference from these conclusions is that degradation of the heterologous 5′ part of the transforming DNA occurs post-synaptically. It can be envisaged that after integration of the homologous part of a molecule in the anchor region the heterologous tail remains accessible to RecJ and perhaps other exonucleases until its illegitimate fusion ensures its protection and inheritance.

Two observations suggest that RecJ has also a role in the pre-synaptic degradation of single-stranded DNA in the cell, although this is a minor role. First, fully homologous transformation with small genomic fragments of 1.5 kb covering the selected *trp*$^+$ gene was increased about 1.5-fold in the *recJ* mutant, an increase which was not significant but observed repeatedly (Kickstein *et al*., 2007). Considering that a strand break in transforming duplex DNA is required to start DNA uptake (Dubnau and Provvedi, 2000; Chen and Dubnau, 2004), only portions of the 1.5 kb DNA pieces are internalized being on average only a few hundred nucleotides long so that even limited exonucleolytic degradation could destroy the genetic marker. Second, in experiments reported here (Table 3) DNA molecules having a very short heterologous tail of 69 nucleotides (the BstBI-digested pBlue-Km1 DNA with 18 nucleotides following the *nptII*$^+$ sequence) were integrated 61-fold less frequent in the wild type than in the *recJ* strain (Table 3). With DNA molecules having longer heterologous tails (e.g. 173 nucleotides after *nptII*$^+$ or more) the decrease of transformation in wild type compared with *recJ* was less severe. If limited pre-synaptic degradation occurs, a genetic effect (loss of marker) would be seen only when the marker is located close to the end of the DNA as was the case with the BstBI-cleaved pBlue-Km1 DNA. In addition, taken up strands from this DNA would most likely have a RecJ-sensitive 5′ end at the 69 nucleotides tail because cytoplasmic DNA entry in *A. baylyi* occurs with the 3′ end ahead (de Vries and Wackernagel, 2002) from the primary DNA break which with these molecules must have been in front of the *nptII*$^+$ gene. In this context it is noteworthy that recently Dutra *et al*. (2007) reported that in *E. coli* RecJ along with exonuclease I also pre-synaptically degrades short oligonucleotides either present as tails or displaced strands on duplex DNA or free as single-strands and thereby reduces homologous recombination and gene conversion. It remains open why in *A. baylyi* pre-synaptic degradation is limited while post-synaptic degradation can extend over hundreds of nucleotides (Fig. 2, compare A with C). Perhaps the cytoplasmic proteins associated with taken up DNA in competent cells and providing protection against DNases *in vitro* (Morrison and Mannarelli, 1979; Vijayakumar and Morrison, 1986) inhibit pre-synaptic degradation by RecJ. This notion would imply that these proteins are no longer present on the DNA after synapsis in the anchor region has occurred in order to allow post-synaptic degradation of the heterologous end. It may be speculated that the homologous pairing in the anchor region stimulates removal of these proteins from short heterologous tails. Removal may not continue to the end of long tails giving them extended protection which would explain the relatively large fraction of distal fusions in the wild type.

A striking result was that HFIR events in *recJ* had the homologous 3′ strand integrated with high preference. Because in the wild type RecJ suppresses HFIR with such integrations leading to enrichment of 5′ integrations, the result with the *recJ* mutant would indicate that in *A. baylyi* homologous strand transfers occur with a strong preference for 3′ strand invasion. This *in vivo* result contrasts with the *in vitro* finding of about equally efficient strand invasion by 3′ and 5′ ends through RecA of *E. coli* (McIlwraith and West, 2001) which was also noted in *E. coli in vivo* studies (Razavy *et al.*, 1996). Either, the 3′ end is more reactive than the 5′ end in the homologous strand invasion in *A. baylyi* or secondary steps to homologous strand invasion preferentially stabilize *in vivo* those heteroduplex regions which were formed by 3′-homologous ends. It is likely that such stabilization could result from the extension of the 3′ end after invasion of the recipient genome by a repair DNA polymerase. Such a possibility has been previously discussed (Kowalczykowski, 2000; de Vries and Wackernagel, 2002) but experimental support needs to be provided. Alternatively, degradation of the displaced 3′ strand could stabilize the joint molecule (Friedman-Ohana and Cohen, 1998; Viswanathan and Lovett, 1998). The mostly 3′-homologous strand integrations in the *recJ* mutant resembles the situation in wild-type HFIR transformant cells of *P. stutzeri* in which these integrations amounted to 98% (Meier and Wackernagel, 2003). An alternate explanation for preferred 3′-homologous strand invasion would be that illegitimate fusion of the 5′ end is favoured relative to illegitimate 3′ end fusion. Our characterization of the nucleotide sequences surrounding the illegitimate fusion sites (extended microhomologies) in wild type and *recJ* did not reveal specific sequence requirements for 5′-illegitimate fusions (V. Schön, K. Harms and W. Wackernagel, unpublished).

The UV-induced formation of Lambda *bio*-transducing phages by illegitimate recombination in *E. coli* is reduced three- to 10-fold in a *recJ* mutant (Ukita and Ikeda, 1996). It was proposed that RecJ degraded one strand at duplex DNA ends during unwinding by a helicase and thereby would expose short nucleotide sequences that could hybridize with direct short repeats leading to illegitimate duplex DNA end joining. This proposal was congruent with the finding that *recJ* deficiency decreased mainly the illegitimate recombination at a hot spot of nine direct repeat nucleotides in Lambda and *E. coli* DNA. In contrast, the spontaneous formation of genomic deletions in the *tonB* and *tonB-trp* regions was not influenced by RecJ which led to the conclusion that UV-induced Lambda *bio* formation and spontaneous genome deletions involve different mechanisms (Mashimo *et al.*, 2003). Our data show that opposite to the observations with Lambda *bio* formation (Ukita and Ikeda, 1996) RecJ suppresses illegitimate recombination when single-stranded DNA forms illegitimate fusions with duplex resident DNA. Thus, the mechanism of illegitimate fusion formation is different during natural transformation and UV-induced duplex DNA end joining.

The RecJ activity level of cells influenced both the frequency of foreign DNA integration events and the average amount of foreign DNA integrated per event (Table 3). This influence is stronger and different from the effect of *mutS* deficiency on HFIR. In *P. stutzeri* and *S. pneumoniae mutS* did not increase the HFIR frequency (Prudhomme *et al.*, 2002; Meier and Wackernagel, 2005), but in the *P. stutzeri* mutant fusion points were somewhat shifted away from the anchor leading to higher amounts of acquired DNA in transformants with a net gain and more often the fusion was outside of a microhomology (Meier and Wackernagel, 2005). In *A. baylyi* the frequency of HFIR events increased from cells with *recJ* overexpression over the wild type to the *recJ* mutant about 40-fold. Although the extent of foreign DNA acquisition per event decreased about eightfold, there was a fivefold higher amount of total acquired foreign nucleotides per population without *recJ* expression. In the light of genetic adaptation the exchange of small DNA segments might be important when for example the modular modification of protein domains is advantageous (Davis *et al.*, 2001; Vogel *et al.*, 2005; Alm *et al.*, 2006) while exchange of large segments would be helpful in acquiring adapted genes or operons (Koonin *et al.*, 2001). Our data show that RecJ contributes strongly to the suppression of short segment integration and has a much less strong regulatory effect on long segment integration.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The two *A. baylyi* strains employed in this study were the parental ('wild-type') strain JV28 [BD413 *trpE*27 *alkM*::(*nptII′* tg4) Rif^R (rifampicin resistance)] (de Vries *et al.*, 2003) and its *recJ* null mutant derivative EK4 [BD413 *trpE*27 *alkM*::(*nptII′* tg4) Rif^R Δ*recJ*] (Kickstein *et al.*, 2007). In EK4 the *recJ* open reading frame has been deleted except for the 16 3′-terminal codons. The RecJ-overproducing strain was JV28 with pEKX2 containing the $recJ^+$ open reading frame including ribosome binding site. The $recJ^+$ DNA fragment was amplified by PCR using the primers recJ-ORF-f2 (ttcccgggTGATGC CATAAATGCGCACAATTC) and recJ-ORF-r2 (aacccgggT TATGTCTGATCTAAATGTAAGATGC; lowercase: non-homologous tail with a *Cfr*9I site), digested with *Cfr*9I and cloned into the singular *Cfr*9I site of pQLICE, a broad host range plasmid vector derived from RSF1010 (Bagdasarian *et al.*, 1981) having also the $lacI^q$ gene (Accession number EF189157). In pEKX2, the $recJ^+$ gene is under control of a synthetical tac promoter lac^{-1} repressor and thus IPTG-inducible (de Boer *et al.*, 1983). The cloning steps were carried out by standard procedures (Sambrook *et al.*, 1989).

The plasmid DNA used in the natural transformation experiments was pBlue-Km1 containing an *nptII*$^+$ gene followed by foreign DNA for HFIR experiments, and pBlue-Km-tg4 which harbours an *nptII*$^+$ followed by homologous DNA (tg4 transcriptional terminator) for homologous recombination (Fig. 1A). The plasmids were isolated from *E. coli*. Bacterial strains were grown in Luria–Bertani (LB) broth or plated on LB medium at 30°C (Sambrook *et al*., 1989). For growth of strains with pEKX2, streptomycin (20 μg ml^{-1}) was present in the medium.

Preparation of heteroduplex DNA

The plasmid pBlue-Km1 (Fig. 1A; de Vries and Wackernagel, 2002) was utilized to produce heteroduplex DNA *in vitro*. In a first step, a new restriction site for BseRI was introduced at the 3′ terminus of *nptII*$^+$ by inverse PCR. Secondly, restriction by BseRI and BstBI was used to remove a segment of 45 base pairs (covering the terminal 29 nucleotides of the 3′ end of *nptII*$^+$ and 16 nucleotides further downstream). In a third step, the fragment was substituted by a synthetic double-stranded oligonucleotide having extensions corresponding to the cohesive ends of the BseRI- and BstBI-cleaved DNA and phosphorylated 5′ ends. The oligonucleotide generated a new BseRI site and inactivated the expression of *nptII*$^+$ by forming a nonsense triplet. The resulting plasmid was termed pVS1. The pVS1 DNA was digested with BseRI which removed the synthetic DNA. The large fragment was purified by agarose gel electrophoresis and subsequently ligated to a duplex DNA fragment obtained by hybridization of two oligonucleotides of 45 base pairs with phosphorylated 5′ ends having two mismatched base pairs. The cohesive ends of the duplex oligonucleotide matched the two overhangs of the BseRI-cleaved pVS1 DNA. This ligation step created pBlue-Km1-tag which was identical to pBlue-Km1 except for the two mismatched base pairs located three nucleotides downstream of the stop codon of *nptII*$^+$ (Fig. 1C). The DNA was digested with ScaI, the linear plasmid was purified by agarose gel electrophoresis and used for transformation.

Natural transformation of A. baylyi

The preparation of competent cells and the transformation assays were as described previously (de Vries and Wackernagel, 1998). Briefly, *A. baylyi* was grown in a shaker in LB broth at 30°C to 1×10^9 cells ml^{-1} and stored at –80°C as a concentrated stock (1×10^{10} cells ml^{-1}) in LB with 10% glycerol until use. When JV28 carried pEKX2, 1 mM IPTG was present during preparation of competent cells as inductor of the *recJ*$^+$ gene. For transformation, a freshly thawed bacterial suspension was diluted to 2.5×10^8 cells ml^{-1} in 1 ml LB containing 100 ng ml^{-1} linear donor DNA, aerated for 90 min at 30°C and plated on LB medium (recipient titre) and LB with 10 μg ml^{-1} kanamycin (transformants). The colonies were counted after 16 h at 30°C. Transformation frequencies were calculated as transformants per recipients and were corrected for the relative competence of the cells used. The relative competence of each batch of competent cells was determined by transformation with chromosomal *trp*$^+$ DNA (100 ng ml^{-1}). *trp*$^+$ colonies were determined on M9 minimal medium (Sambrook *et al*., 1989) containing 10 mM succinate after 40 h incubation at 30°C. The rare kanamycin-resistant transformants that resulted from plasmid cointegrate formation (due to incomplete linearization of plasmid DNA by restriction enzyme digestion) were identified by their resistance against ampicillin (50 μg ml^{-1}) and were not included in the calculations. Without pBlue-Km1 or pBlue-Km1-tag DNA no kanamycin-resistant colonies were obtained.

Characterization of HFIR transformants

Kanamycin-resistant HFIR transformants were randomly taken from at least six independent transformation experiments per strain tested to determine their fusion sites by sequencing. The DNA segment containing the illegitimate fusion was amplified by PCR using the forward primer F1 (ATTCGCAGCGCATCGCCTTC; binding site in the part of *nptII* that is absent in the recipient) and one of the following reverse primers (complementary to the recipient DNA; see Fig. 2): R2 (CATGCACATACAAATGGACG), R5 (CTGAAT GTCGTTGTAACTGG), or R9 (GATGGCGTACAGCTACT TGG) respectively.

The DNA strand integrated during HFIR was identified in randomly chosen HFIR transformants obtained with heteroduplex DNA by two PCR tests using strand tag-specific primers: the primers npt7–26 (GAACAAGATGGATTGC ACGC) plus GG2 (TTCGAACCCCAGAGTCGG) for 3′-homologous integration and npt7–26 plus TT2 (TTCG AACCCCAGAGTCTT) for 5′-homologous integration.

The PCR screening for distal fusion sites in HFIR transformants was performed using the primers F2 (CATCCATGC-CCCCGAACTGC) and R9 (Fig. 2). If a product was obtained, the transformant was classified as having a distal fusion site. If no product was obtained, the transformant was classified as having a proximal fusion which was verfied by a second PCR using primers F1 and R5 or R9 (Fig. 2). PCR reactions were carried out using *Taq* (Molzym, Bremen, Germany) or Phusion DNA polymerase (Finnzymes, Espoo, Finland) according to the manufacturers' instructions. When Phusion was used, dimethylsulfoxide (10%) was included in the reaction mix.

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