Frequent integration of short homologous DNA tracks during *Acinetobacter baylyi* transformation and influence of transcription and RecJ and SbcCD DNases

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The minimal length of integrated homologous donor DNA tracks in *Acinetobacter baylyi* transformation and factors influencing the location and length of tracks were determined. Donor DNA contained the *nptII* gene region (kanamycin resistance, Km^R). This region carried nine approximately evenly spaced silent nucleotide sequence tags and was embedded in heterologous DNA. Recipient cells carried the normal *nptII* gene with a central 10 bp deletion (kanamycin-sensitive). The Km^R transformants obtained had donor DNA tracks integrated covering on average only 4.6 (2–7) of the nine tags, corresponding to about 60 % of the 959 nt homologous donor DNA segment. The track positions were biased towards the 3' end of *nptII*. While the replication direction of recipient DNA did not affect track positions, inhibited transcription (by rifampicin) shifted the beginning of tracks towards the *nptII* promoter. Absence of the RecJ DNase decreased the length of tracks. Absence of SbcCD DNase increased the integration frequency of the 5' part of *nptII*, which can form hairpin structures of 43–75 nt, suggesting that SbcCD DNase interferes with hairpins in transforming DNA. In homology-facilitated illegitimate recombination events during transformation (in which a homologous DNA segment serves as a recombinational anchor to facilitate illegitimate recombination in neighbouring heterologous DNA), on average only about half of the approximately 800 nt long tagged *nptII* anchor sequences were integrated. From donor DNA with an approximately 5000 nt long homologous segment having the *nptII* gene in the middle, most transformants (74 %) had only a part of the donor *nptII* integrated, showing that short track integration occurs frequently also from large homologous DNA. It is discussed how short track integration steps can also accomplish incorporation of large DNA molecules.

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INTRODUCTION

Natural genetic transformation has been recognized as a major mechanism of horizontal gene transfer among prokaryotes occurring in their natural environment and has been experimentally studied in strains from many taxonomic groups of bacteria and archaea (Lorenz & Wackernagel, 1994; Chen & Dubnau, 2004; de Vries & Wackernagel, 2005; Thomas & Nielsen, 2005). Transformation involves the binding of duplex donor DNA to the DNA-uptake competent cell, and, after introduction of a double-strand break, the transport of one single strand into the cytoplasm, where it can be integrated into resident DNA by homologous recombination (Lacks, 2000; Chen & Dubnau, 2004). Studies following the fate of radioactively and/or density-labelled

donor DNA revealed that on average fragments of about 6000 nt were integrated during transformation of *Streptococcus pneumoniae* (Gurney & Fox, 1968), 8500–10 000 nt in *Bacillus subtilis* (Bodmer & Ganesan, 1964; Dubnau & Cirigliano, 1972), and 8400 nt in *Haemophilus influenzae* (Notani & Goodgal, 1966). Electron microscopic studies showed in *B. subtilis* the integration of an average length of about 10 000 nt with an upper limit of 30 000 nt (Fornili & Fox, 1977). For the measurement of integrated segments below 1500 nt these techniques were less suited (Bodmer, 1966; Gurney & Fox, 1968; Fornili & Fox, 1977). Genetic mapping in *S. pneumoniae* indicated an average length of about 500 nt integrated in transformants and it was suspected that the biochemical studies overestimated the amount of DNA recombined into the genome per transformation event (Ephrussi-Taylor & Gray, 1966). This apparent conflict has not been addressed in subsequent studies.

Abbreviations: HFIR, homology-facilitated illegitimate recombination; MMR, mismatch repair.

A recent study suggested that natural genetic transformation in *Acinetobacter baylyi* may also be elicited by the integration of rather short DNA stretches. It was observed that short homologous DNA segments of 183–1096 nt (termed anchor) in otherwise heterologous DNA serve as places for homologous recombination during transformation and strongly facilitate illegitimate recombination in the neighbouring heterologous DNA, leading to the integration of foreign DNA stretches (de Vries & Wackernagel, 2002). This phenomenon, termed homology-facilitated illegitimate recombination (HFIR) was also observed in *S. pneumoniae* (Prudhomme *et al.*, 2002) and *Pseudomonas stutzeri* (Meier & Wackernagel, 2003), involving similarly short anchor segments. In these studies it remained open whether the whole anchor segment was recombined into the transformants or only a part of it.

Here we have combined genetic and molecular techniques to examine in individual natural transformants of *A. baylyi* the lengths of the homologous DNA stretches integrated to the left and right of a selective 10 bp marker. We used DNA with dispersed nucleotide sequence tags as donor DNA to trace its integration in the recipient cell. We found that from homologous DNA segments of about 1000 or 5000 nt in length mostly relatively short tracks of a few hundred nucleotides were integrated. The lengths and positions of the integrated tracks relative to the central marker were influenced by transcription of resident DNA and the presence of the single-strand DNases RecJ and SbcCD. In HFIR events also generally only a part of the relatively short homologous anchor segment was integrated.

METHODS

Bacterial strains, plasmids and growth conditions. *A. baylyi* strains used for natural transformation are listed in Table 1. Parental

strains for double mutants were EK4 ($\Delta recJ$; Kickstein *et al.*, 2007) and KOM17 ($\Delta sbcCD$; Harms & Wackernagel, 2008). Cells were grown and plated using LB medium (Sambrook *et al.*, 1989) at 30 °C. *Escherichia coli* DH5 α (Hanahan, 1983) was used during construction of plasmids, which were introduced into the strain by electroporation as reported by Dower *et al.* (1988). If required, media contained kanamycin (10 $\mu\text{g ml}^{-1}$), tetracycline (10 $\mu\text{g ml}^{-1}$) and/or streptomycin (30 $\mu\text{g ml}^{-1}$).

Construction of strains and plasmids. *A. baylyi* strain JV18 is a *mutS* deletion mutant in which base pairs 1–1083 of the ORF (2646 bp; Barbe *et al.*, 2004) are deleted (constructed and provided by J. de Vries, University of Oldenburg). Into this background other deletion alleles were crossed in by a two-step procedure involving, first, transfer of a substitution mutation of the gene (with an *nptII* cassette marker) followed by removal of the marker through a second transfer as described (Harms *et al.*, 2007; Kickstein *et al.*, 2007), resulting in the *recJ mutS* and *sbcCD mutS* double-deletion mutants (Table 1). Plasmid pRKNH3- Δi (Hülter & Wackernagel, 2008), derived from pRK415 (Keen *et al.*, 1988; accession no. EF437940), carries a cassette consisting of *bla*⁺, *nptII* Δi and *tetA*⁺. Plasmid pRKNH3-II- Δi , having the *bla*⁺ *nptII* Δi *tetA*⁺ cassette in the opposite orientation, was constructed by removing the *StuI*–*EcoRV* fragment encompassing *tetA*⁺ from pRKNH3 (accession no. EF621523) and replacing it by a PCR fragment obtained with the primers #8 (5'-GCGTTTGCAGGGCCATAGACG-3') and #14 (5'-GCTAGGGCCTTGTGGGGTCAG-3') with pRKNH3- Δi as template and which covered *bla*⁺, *nptII* Δi and *tetA*⁺. A clone with the desired orientation was chosen. Plasmid pN3 carrying the *nptIIm* region (see Results) was derived from the pACYC184-based plasmid pN1 (Hülter & Wackernagel, 2008). The *nptIIm* region, synthesized and cloned in the plasmid vector provided by the manufacturer (TOP Gene Technologies), was cut out with *AscI* and *SacI* and ligated to the *AscI*- and *SacI*-treated PCR product obtained from pN1 by inverse PCR using the primers #22 (5'-AGggcgccAGCTGGCAATTC-3') and #23 (5'-CTTctgagctCGACTCTGGGG-3'), which generated the restriction sites for *AscI* and *SacI* (added nucleotides shown by italics; *AscI* and *SacI* sites, lower case) to the backbone of pN1. Plasmid pN4 containing a 4.9 kbp region of pRKNH3- Δi (but with the *nptIIm* region) was constructed as follows. The PCR product of the *nptIIm* region of pN3 obtained using primers f1 (5'-GTAAAGCGAACC-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source
<i>A. baylyi</i>		
ADP1	<i>trpE27</i>	Juni (1972)
JV18	$\Delta mutS$ <i>trpE27</i>	J. de Vries
NH7	$\Delta mutS$ $\Delta recJ$ <i>trpE27</i>	This work
NH20	$\Delta mutS$ $\Delta sbcCD$ <i>trpE27</i>	This work
Plasmids		
pRKNH3- Δi	Derivative of pRK415 containing <i>bla</i> ⁺ <i>nptII</i> Δi <i>tetA</i> ⁺ (9.4 kbp)	Hülter & Wackernagel (2008)
pRKNH3-II- Δi *	Derivative of pRKNH3- Δi	This work
pRKNH3- ΔL	Derivative of pRK415 containing the 5'-truncated <i>nptII</i>	Hülter & Wackernagel (2008)
pRKNH3- ΔR	Derivative of pRK415 containing the 3'-truncated <i>nptII</i>	Hülter & Wackernagel (2008)
pN3	Derivative of pN1 containing the <i>nptIIm</i> region having nine sequence tags (5.6 kbp)	Hülter & Wackernagel (2008); this work
pN4	Derivative of pN3 with the <i>nptIIm</i> region flanked by <i>bla</i> ⁺ and <i>tetA</i> ⁺ (9 kbp)†	This work

*The *bla*⁺ *nptII* Δi *tetA*⁺ cassette was inverted compared to pRKNH3- Δi .

†The homologous region to pRKNH3- Δi extends over 4931 bp.

GGAATTGCCAGCTG-3') and #47 (5'-TCGAACCCAGAGTCG-AGCTC-3') was cloned into the *AleI* site of pRKNH3-null (Hülter & Wackernagel, 2008) between *bla*⁺ and *tetA*⁺, giving rise to pRKNH3-*nptII*m. From this plasmid the 4.9 kbp *SfiI*-*NsiI* fragment carrying *bla*⁺, the *nptII*m region, and *tetA*⁺ was cut out and ligated to the 4.1 kbp *SfoI* vector backbone fragment of pN1. All constructs and plasmids were verified by sequencing.

Natural transformation. Competent cells were prepared and their competence was tested as described by Harms *et al.* (2007). Transformation with DNA of plasmids pN3 or pN4 (100 ng ml⁻¹) linearized by *XbaI* (cutting opposite to the *nptII*m region) was performed at 30 °C with aeration for 90 min in LB medium. Transformants were counted after 16–20 h at 30 °C on medium containing kanamycin. Total cell count (c.f.u.) was determined on LB. Transformation frequencies are given as transformants per c.f.u. of recipient cells. The rifampicin concentration suitable to inhibit transcription during transformation was determined using *A. baylyi* with a plasmid derivative of pQLICE (accession no. EF189157) carrying an autorepressed *lacI*^q-*tac* promoter-*lacZ* cassette. In competent cells of this strain, β -galactosidase activity (Miller, 1972) induced by IPTG (1 mM) was reduced depending on the rifampicin concentration present during induction. At 2.5 μ g ml⁻¹ the reduction was 40 %, at 5 μ g ml⁻¹ 91 %, at 10 μ g ml⁻¹ and at 20 μ g ml⁻¹ greater than 99 %. The recovery of gene expression and cell growth following removal of rifampicin by sedimentation of cells and incubation in fresh medium requires considerable time spans (Stubbings *et al.*, 2006). At 5 μ g ml⁻¹, growth recovery of competent *A. baylyi* cells occurred within 1 h at 30 °C, at 10 μ g ml⁻¹ and 20 μ g ml⁻¹ not before 6 h. For transformation with inhibited transcription, rifampicin (5 μ g ml⁻¹ final concentration) was added together with the DNA to competent cells. After 90 min at 30 °C the cells were sedimented, resuspended in LB, and aerated at 30 °C for 60 min to allow gene expression and growth recovery in the absence of rifampicin.

Characterization of transformants. Randomly picked transformants from at least two independent experiments were purified by three consecutive single-colony isolation steps on selective medium. The presence of donor DNA sequence tags in the Km^R transformants was determined by first amplifying the *nptII* region by PCR and then testing for cleavage by restriction endonucleases specific for the nine tags (*AscI*, *BamHI*, *MfeI*, *PstI*, *SmaI*, *Sall*, *NcoI*, *EcoRV* and *SacI*). In the case of transformants with a filled-up 10 bp deletion in *nptII* Δ i the left and the right parts of the *nptII* region were separately amplified with primers #30 (5'-CAATGCGGCGCTGCATACG-3') and #31 (5'-CCGATCAAGCGTATGCAGC-3'), binding at the site of the filled-up deletion (Δ i) in opposite orientations, and primers binding in the 5'-terminal region of *nptII* (#5, 5'-GTCAGGCAACTATGGATGAAC-3') and the 3'-terminal region (#25, 5'-GCAACGAGCCGATCGCTGATC-3') (see Fig. 1b). PCR products of the restored *nptII* genes in HFIR transformants obtained from strains with pRKNH3- Δ L or pRKNH3- Δ R, respectively, were obtained with primers binding in the filled-up terminal deletions (#26, 5'-CTGCAAAGTAACTGGATGGC-3' for Δ L; #21, 5'-GAGCTCAGAAAGACTCGTCAAGAAGG-3' for Δ R) and reverse primers located on the opposite end of *nptII* (#5 and #25).

Description of integrated DNA tracks and statistical analyses. Integrated donor DNA tracks in transformants were identified by the presence of contiguous sets of nucleotide tags. Depending on the experiment, either the number of tags to the left of the filled-up 10 bp deletion, the number to the right, or the total number of tags was considered. We used the two-tailed Mann-Whitney *U*-test to judge whether the distributional patterns of tags in transformants differed from each other between compared strains, or in experiments with and without rifampicin treatment. The Wilcoxon signed-rank test was employed to test if within a strain the pattern of tag presence on the

Fig. 1. The *nptII*m gene region and the experimental strategy. (a) The nine positions of nucleotide exchanges (tags) in *nptII*m and the exchanges (plus nucleotide number) compared to *nptII*⁺ (WT) are shown. Nucleotide no. 1 is the first nucleotide of the homologous DNA segment, nucleotide no. 959 the last. The *nptII* promoter is shown as a kinked arrow. The ORF of *nptII*m (bold arrow) and the restriction sites generated or eliminated are indicated. The borders between the homologous segment (thick line) and the flanking heterologous DNA (thin line) are indicated by short vertical lines. (b) Schematic result of a recombination of *nptII*m donor DNA and *nptII* Δ i (10 bp deletion: white box) in the recipient cell. The 11 intervals between tags or between tags and the 10 bp sequence unique to *nptII*m are numbered from left to right and are given with their lengths (number of nt). The two formal crossover events (X) in the homologous region (grey shaded; 509 nt to the left and 440 nt to the right of the deletion) giving a Km^R transformant and the approximate extension of the integrated donor DNA track (line below ORF) are shown. Pairs of open and filled arrows mark positions and orientations of primers used to amplify right and left parts of the *nptII* region.

left side differed from that on the right side. In HFIR transformants only the tags located outside of the filled-up deletion (7 in Δ L and 8 in Δ R) were considered.

RESULTS

The genetic system

To determine the length of homologous DNA tracks integrated during natural transformation, the *nptII* gene

determining Km^R was the selective marker to identify transformants. Recipient cells carried an *nptII* gene region including promoter having a 10 bp deletion roughly in the middle of the ORF (*nptII* Δ i; Km^S). In most experiments the *nptII* Δ i was located on pRKNH3- Δ i (Table 1). The donor DNA carried a functional *nptII* gene region (959 bp; Km^R) and was in most experiments *Xba*I-linearized plasmid pN3, providing heterologous flanks of about 2.3 kbp to *nptII*. The donor *nptII* gene region contained silent nucleotide exchanges (tags) at nine roughly evenly spaced positions (Fig. 1a) and was termed *nptII*m. Seven of the exchanges created new restriction sites and two eliminated sites present in *nptII* $^{+}$ (Tn5; Beck *et al.*, 1982). The tags were employed to discriminate between donor and recipient DNA in transformants. Fig. 1(b) shows an example of how two formal recombination events produce a Km^R transformant of the recipient cell. The tags present in the DNA of the transformant are indicative of the track of integrated donor DNA, covering in this case two intervals to the left of Δ i and two to the right (Fig. 1b). During strand integration the heteroduplex formation will lead to mismatched base pairs at sequence tags. Mismatches are known to be the target of the mismatch repair (MMR) system aborting recombination (for review see Modrich & Lahue, 1996). Therefore, the recipient cells always carried a *mutS* deletion eliminating the mismatch recognition component of the MMR system (Modrich & Lahue, 1996).

Restriction analysis was performed with two PCR products, each covering one half of the *nptII*m region of individual transformants. These products were obtained with two primer pairs, of which one primer each bound to the filled-up 10 bp deletion in the Km^R transformants (in opposite orientations; Fig. 1b) and the corresponding other primer outside of the *nptII* region (Fig. 1b). This procedure restricted amplification to sequences that were the product of recombination between the *nptII* Δ i region and the donor *nptII*m sequence by targeting the 10 bp sequence that is unique to *nptII*m. The distinction was necessary because amplification of background *nptII* Δ i sequences was seen in about 25 % of the purified Km^R transformants. This unexpected observation may be explained by the fact that pRKNH3- Δ i is probably located in multiplasmid clusters that replicate and partition as units, as has been shown for its parent RK2 (Pogliano *et al.*, 2001). The ‘mixed’ transformants always contained a single recombinant sequence type (determined by cloning and sequencing), indicating that the level of ongoing recombination is minor. The restriction analysis was highly reliable as its results were fully confirmed by sequencing of the PCR products of 35 transformants from different experiments.

Length distribution of integrated DNA tracks within *nptII*

With the *mutS* mutant JV18 carrying pRKNH3- Δ i, Km^R transformants were obtained with *Xba*I-linearized pN3 at a

frequency of $2.8(\pm 0.5) \times 10^{-3}$ ($n=3$), which was indistinguishable from that obtained with the *nptII* $^{+}$ gene (plasmid pN1) having no sequence tags (3×10^{-3} ; Hülter & Wackernagel, 2008). In a corresponding *mutS* $^{+}$ strain the transformation frequency was almost three orders of magnitude lower [$3.6(\pm 0.6) \times 10^{-6}$; $n=3$], confirming the recombination-suppressing function of MMR (Modrich & Lahue, 1996).

Analysis of 43 JV18 transformants for the presence of donor DNA tags revealed that each contained one continuous track of donor DNA and that none of them had the complete homologous *nptII*m region integrated (Fig. 2a). On average 4.6 of the nine tags were present, with the shortest tracks having two and the longest having seven tags. Considering that hybridization and ligation for strand-joining requires some extra nucleotides beyond the terminal tags (mismatches) per track, the mean number of 4.6 tags represents about 60 % of the 959 nt homologous stretch. The distribution of strand-joining events to the 11 intervals (Fig. 1b) was biased towards the 3' end of *nptII* (Fig. 2a). With the filled-up deletion as the reference point, the number of tags integrated to the left in the 43 transformants (mean: 1.8 tags) was significantly smaller than that integrated to the right (mean: 2.8; Wilcoxon signed-rank test; $P<0.01$). The bias could result from replication, which for the orientation of the RK2-derived recipient plasmid shown in Fig. 1(a) runs from the left to right. As a test the *nptII* Δ i cassette in the resident plasmid was inverted (giving pRKNH3-II- Δ i) so that replication entered *nptII* Δ i from the right side. The pattern of tag integrations in 45 transformants of JV18/pRKNH3-II- Δ i was indistinguishable from that in Fig. 2(a) (data not shown) and also the mean number of tags integrated (4.5) was not changed, excluding an influence of the replication direction on the track pattern.

For a control experiment, the *nptII* Δ i gene was also integrated into the chromosome of JV18 by use of a gene-targeting vector as described by de Vries *et al.* (2003), putting the fragment with the *nptII* Δ i ORF (849 bp) downstream of p_{tac} in the non-essential *alkM* gene of *A. baylyi*. Using linearized pN3 as donor DNA, Km^R transformants with this strain were never ‘mixed’ (see above), appeared at a frequency as high as with strain JV18/pRKNH3- Δ i (confirming the equal transformation efficiency with the target on a plasmid or the chromosome; de Vries *et al.*, 2003), and had DNA tracks of similar length integrated (Fig. 2b), suggesting that the frequent short track integration is not due to the target sequence being located on a plasmid.

Transcription affects the position of the strand-joinings

We considered that transcription from the promoters of *nptII* (Fig. 1b) and the preceding *bla* $^{+}$ in pRKNH3- Δ i (Hülter & Wackernagel, 2008) could affect the location of strand-joinings. Therefore, the transformation experiment

of Fig. 2(a) was repeated under conditions of transient inhibition of transcription by rifampicin ($5 \mu\text{g ml}^{-1}$), which reversibly interferes with transcription in the cells (Stubbings *et al.*, 2006; see Methods). In the transformants (Fig. 3a) the mean number of donor DNA tags present on the left side of the filled-up deletion was significantly increased from 1.8 (without rifampicin) to 2.5 (Mann–Whitney *U*-test; $P < 0.01$). The mean total number of tags

Fig. 2. Donor DNA tracks present in Km^R transformants of *A. baylyi* strains from three different experiments. The top lines show the donor DNA with the *nptII* regions including the numbering of intervals as in Fig. 1(b). The vertical mark with an asterisk indicates the position of the 10 nt to fill up the deletion in *nptII* Δ i. The dotted vertical lines give the positions of the tags (Fig. 1a). The homologous parts of donor DNA are shown as thick lines, the heterologous as thin lines. The donor DNA tracks present in transformants are shown below the top lines as thick dark lines. The ends of the integrated tracks are positioned in the middle of the interval in which strand-joining had occurred. The homologous DNA not replaced is shown in grey. (a) Donor DNA was linearized pN3; the target *nptII* Δ i region was located on pRKNH3- Δ i (region homologous with donor DNA: 949 nt); (b) donor DNA was linearized pN3; the target fragment with *nptII* Δ i was located in the chromosomal *alkM* gene (region homologous with donor DNA: 849 nt); (c) donor DNA was linearized pN4; the target *nptII* Δ i region was located on pRKNH3- Δ i (region homologous with donor DNA: 4931 nt). The total numbers of analysed transformants were 43 in (a), 40 in (b) and 30 in (c). The scale for the homologous region between donor and recipient DNA is given in nucleotides, with the position 0 indicating the centre of the filled-up 10 nt deletion in transformants.

present was 4.8 with rifampicin (4.6 without). The result indicates that inhibition of transcription shifts the strand-joinings on the left side closer to the promoter.

RecJ deficiency decreases the integrated track length

The RecJ protein of *A. baylyi* (ACIAD3500) corresponds to the 5' specific single-strand exonuclease RecJ of *E. coli* involved in homologous recombination (Kickstein *et al.*, 2007). To test if RecJ influences the homologous track integration, we used the *recJ mutS* double mutant NH7/pRKNH3- Δ i as recipient. The transformation frequency of $7.2 (\pm 0.3) \times 10^{-4}$ ($n=3$) was about fourfold lower than that of the *recJ*⁺ strain [$2.8 (\pm 0.5) \times 10^{-3}$ ($n=3$); *t*-test: $P < 0.01$]. Strand-joinings on the right side were shifted towards the filled-up deletion (Fig. 3b) as the number of tags present on this side in the *recJ* transformants (mean: 2.0) was significantly lower than the corresponding number in *recJ*⁺ (mean: 2.8; Mann–Whitney *U*-test; $P < 0.01$). On the left side, a change of the distribution of the strand-joinings was not discernible (see next section) and the mean number of total integrated tags was 3.9 (*recJ*⁺: 4.6; Mann–Whitney *U*-test; $P = 0.077$). The results suggest that RecJ supports transformation of the pRKNH3- Δ i recipient strain and integration of normal length tracks.

Absence of SbcCD DNase stimulates the integration of specific DNA segments

Strand-joinings in the two leftmost intervals of *nptII* were observed only once among 118 transformants (Figs 2a and 3b, and data not shown). We examined the *nptII* region

Fig. 3. Effect of transcription inhibition by rifampicin (a; ▲), *recJ* deficiency (b; ◆) and *sbcCD* deficiency (c; ◇) on the distribution of strand-joinings to the 11 intervals (Fig. 1) leading to track integration. The distribution in the wild-type strain JV18 (■; Fig. 2a) is included in each graph. The numbers of examined transformants were 56 in (a), 30 in (b) and 30 in (c). The scale is given in nucleotides as in Fig. 2.

for the potential to form hairpin structures by using the mfold version 3.2 web server (Zuker, 2003) and identified at the left end three imperfect palindromes (A, B, C; Fig. 3c). Structure A consists of 75 nt [40 contributing to the stem structure and 10 constituting the top loop; $\Delta G^0 = -11.11 \text{ kcal mol}^{-1}$ (46.48 kJ mol $^{-1}$)], structure B consists of 43 nt [30 nt stem and 4 nt top loop; $\Delta G^0 = -7.29 \text{ kcal mol}^{-1}$ (30.50 kJ mol $^{-1}$)], and structure C of 70 nt [44 nt stem, 8 nt top loop; $\Delta G^0 = -11.34 \text{ kcal mol}^{-1}$ (47.45 kJ mol $^{-1}$)]. SbcCD DNase cleaves hairpin structures *in vitro* at the loop–duplex border (Connelly *et al.*, 1999) and also attacks palindromic structures in *E. coli* when forming a hairpin in single-stranded DNA during replication (Chalker *et al.*, 1988; Leach *et al.*, 1997). Cleavage of the loop of structure C would remove all three leftward located tags from the transforming DNA and cleavage of structure B would remove two of them. Employing the *sbcCD mutS* strain NH20/pRKNH3- Δ i as recipient, among the transformants more strand-joinings were observed in the leftmost three intervals (Fig. 3c), with five joinings in intervals 1, three in interval 2, and about 20% more in interval 3, compared to the corresponding *sbcCD*⁺ strain. The presence of the five left tags was significantly increased at the 5% level (chi-squared test; $P=0.021$) in the *sbcCD* strain compared to *sbcCD*⁺, while presence of total tags was not. The results suggest that the SbcCD DNase affects transformation by DNA with the potential to form hairpin structures.

Frequent integration of short tracks from long homologous DNA

To test whether the limited homology in pN3 was the reason for the rather short integrated tracks we used *Xba*I-linearized pN4 (Table 1) as donor DNA, which contains 2353 nt homologous to pRKNH3- Δ i to the left of the selective 10 nt in *nptII*m and 2568 nt to the right. With this DNA the transformation frequency of JV18

[$1.5(\pm 0.6) \times 10^{-2}$; $n=3$] was about fivefold higher than with pN3 DNA, indicating that the longer region of homology stimulated recombinant formation. The frequency of transformants having all tags of the *nptII*m gene region integrated increased from 0% (Fig. 2a) to 27% (8/30; Fig. 2b). Still, the majority of transformants (73%) had only a part of *nptII*m integrated. This part was either internal to *nptII*m (13% of the transformants) or extended into the left homologous flank of *nptII*m (7%) or into the right (53%). Since the pN4 donor DNA fragment with the *nptII*m region (959 nt) roughly in the middle had a length of 9029 nt, the DNA uptake-related cut would occur in approximately 1/10 of molecules (959/9029) within *nptII*m. These cut molecules can evoke only the partial transfer of *nptII*m. As a much larger proportion of transformants (73%) had just a part of *nptII*m integrated, this observation supports the notion of the frequent integration of short DNA tracks.

Distribution of homologous strand-joinings within anchor DNA in HFIR transformants

The *nptII*m region was employed as donor DNA to determine the location of homologous recombination events in the anchor segment during HFIR (see Introduction). Two JV18 recipient strains were used, one with pRKNH3- Δ L, having a 5' terminally deleted *nptII* (lacking the promoter region and 40 nt of the ORF; Km^S), and one with pRKNH3- Δ R, having a 3' terminally deleted *nptII* gene (lacking 51 nt from *nptII* ORF; Km^R). Fill-up of these deletions (leading to Km^R) by transformation with linearized pN3 DNA (Fig. 4) required homologous recombination in the truncated resident *nptII* and illegitimate recombination to the left of the deletion in pRKNH3- Δ L or to the right of the deletion in pRKNH3- Δ R. With both recipient strains only one of 73 Km^R transformants had all tags of the donor anchor DNA integrated. In pRKNH3- Δ L on average 4.2 of the seven tags

Fig. 4. Distribution of strand-joining events in the anchor sequences of HFIR transformants obtained with strains JV18/pRKNH3- Δ L (○; $n=39$) and JV18/pRKNH3- Δ R (●; $n=34$). In the pN3 donor DNA (top) the anchor sequences are shown by dark bold lines and the stretches required for terminal deletion fill-up by grey shaded rectangles. The scales in nucleotides start with 1 for the position of the first nucleotide of the respective anchor sequence.

(anchor length 774 nt) and in pRKNH3- Δ R 4.2 of the eight tags (anchor length 889 nt) were integrated (Fig. 4), corresponding to about half of the anchor segments. The strand-joinings were rather evenly distributed over the anchor segments, with the tag next to the filled-up deletion being present in each transformant. The results show that generally only a part of the anchor region is recombined into recipient DNA during HFIR.

DISCUSSION

In this study transformants had integrated on average only about 60% of the 959 nt homologous donor DNA stretch (*nptII*m) embedded in heterologous DNA. When the *nptII*m was in the middle of an approximately 5000 nt long homologous stretch, the majority of transformants had still integrated only a part of *nptII*m. Results obtained with the *nptII* target sequence on a plasmid were similarly obtained when the *nptII* target was located on the chromosome. Biochemical studies on DNA integration in *A. baylyi* are not available to compare the data with the relatively short integrated DNA tracks observed here. Our results are not in conflict with studies in which by selection the less frequent integration of fragments of over 10 000 nt (Gerischer & Ornston, 2001) or even 50 000 nt (M. Baumgart & J. de Vries, unpublished results) was observed. Our results are in line with the observation that in *S. pneumoniae* transformation, mostly short DNA segments were recombined into the recipient genome (Ephrussi-

Taylor & Gray, 1966) and that biochemical studies mainly reveal the less frequent cases of long segment integrations.

As the DNA molecules used here for transformation were large in comparison to the examined 959 nt *nptII*m stretch (5600 nt and 9000 nt, respectively; Table 1), the uptake-related cut was located mostly outside of the 959 nt stretch. This implies that recombination generally did not start from a homologous end of donor DNA. Such an end can invade homologous duplex DNA with the help of RecA and thereby form a plectonemic joint (Kowalczykowski *et al.*, 1994). Rather, most recombination events seen here apparently initiated from the joining of an internal homologous segment in the otherwise heterologous single-stranded donor DNA with the resident duplex DNA. Such a three-stranded homologous association without any internal DNA end is catalysed by RecA and is termed a paranemic joint (Kowalczykowski *et al.*, 1994). From transformation studies with *S. pneumoniae*, Pasta & Sicard (1999) also concluded that most integrations started from paranemic joints. Further support for this view comes from the recent finding that a sequence homologous to resident DNA within otherwise heterologous DNA can elicit a double illegitimate recombination event to the left and right of the homologous stretch, which leads to the simultaneous integration of two heterologous DNA segments flanking the homologous stretch in transformants (Hülter & Wackernagel, 2008). In these cases the primary donor and recipient DNA association was also presumed to be a paranemic joint. Following the paranemic joint formation, the integration of a homologous donor DNA segment as seen here requires two cleavage events of the donor strand and two of the resident strand to be displaced plus two ligation steps. It is not known which enzyme(s) effects the required cuts for the conversion of the paranemic to a plectonemic joint. The RuvC endonuclease normally acting on four-strand junctions during resolution of Holliday structures has been shown to act also on three-way junctions *in vitro* (Eggleston & West, 2000) and might be a candidate. However, we found that in a Δ *ruvC* mutant the frequency of transformation was not decreased and in a Δ *ruvC* Δ *mutS* strain the pattern of integrated donor DNA tracks in pRKNH3- Δ i was indistinguishable from that observed in the corresponding *ruvC*⁺ strain (Fig. 2; K. Harms, N. Hülter, W. Wackernagel, unpublished results).

The pattern of the integrated tracks was shifted towards the closely located constitutive promoter when transcription was inhibited. Transcription may affect DNA integration if the progressing RNA polymerase itself or the positive superhelical constraint ahead of the transcription complex provide a hindrance to the RecA-catalysed formation and extension of plectonemic or paranemic joints (Liu & Wang, 1987; Honigberg & Radding, 1988; Rahmouni & Wells, 1992). Further downstream of the promoter where the superhelical stress is lower (Rahmouni & Wells, 1992), interactions of donor and recipient DNA may have a higher chance to lead to strand integration. Changes of DNA topology evoked by transcription can affect expres-

sion of upstream and downstream genes (for references, see Wang, 1996), and our results suggest that they also influence recombinative DNA integration during transformation.

RecJ deficiency decreased transformation about fourfold, while in previous studies transformation of a chromosomal marker (*trp*⁺) was not affected (Kickstein *et al.*, 2007). We propose that homologous DNA integration into a resident plasmid as examined here is less effective than into the chromosome and that the RecJ DNase helps to overcome the detriment. In *E. coli* a role of RecJ DNase in homologous recombination is seen in the postsynaptic degradation of the displaced strand so that strand exchange is driven by removal of the competitor strand for pairing (Razavy *et al.*, 1996; Viswanathan & Lovett, 1998). The RecJ DNase of *A. baylyi*, which complements a *recJ* mutant of *E. coli* (Kickstein *et al.*, 2007), could also act in competitor strand removal. This view is strengthened by the shorter DNA tracks integrated in the *recJ* strain (Fig. 3b). Competitor strand removal may be more important when a plasmid instead of the chromosome is the target of donor DNA integration because topological constraints to heteroduplex formation and extension may be higher in the plasmid (Friedman-Ohana & Cohen, 1998; Viswanathan & Lovett, 1998).

In transformants obtained with pN3, the leftmost intervals of *nptII*m DNA with the potential to form hairpin structures were rarely observed in wild-type (<1.1%) and more frequently in *sbcCD* cells (20%), suggesting that hairpin structures may sensitize transforming DNA to SbcCD. The same nucleotide sequences are present in the recipient DNA. There they do not pose a problem, as imperfect palindromes of up to 200 nt are tolerated in bacteria (Warren & Green, 1985; Yoshimura *et al.*, 1986). In the case that they are subject to cleavage by SbcCD, e.g. during replication, the *recA*⁺*recBCD*⁺-dependent recombination can repair the resulting double-strand break (Leach *et al.*, 1997). Such a repair is impossible in a transforming single strand. As transformation-specific proteins bind to the taken-up single-stranded DNA and protect it against DNase action in the cytoplasm (Morrison & Mannarelli, 1979; Morrison *et al.*, 2007; Mortier-Barriere *et al.*, 2007), this protein coverage may normally prevent hairpin formation and/or hairpin cleavage by SbcCD. If these proteins are released from DNA regions preparing for plectonemic or paranemic joint formation, this could lead to localized hairpin formation and thus sensitization to SbcCD. Such an effect could explain why the intervals with hairpin-forming potential were more frequently integrated from DNA having a large (5000 nt) homologous region (Fig. 2c) in which recombination can occur away from the critical intervals. It was recently observed that transforming DNA molecules were degraded by RecJ DNase preferentially at the end close to the recombination event, which was attributed to the local removal of transformation-specific protecting proteins (Harms *et al.*, 2007). It is possible that the hairpin-forming potential of *nptII*m contributed to the integration of short

tracks, but this contribution cannot be large as the number of total donor DNA tags integrated was not significantly increased by the *sbcCD* mutation.

Essentially all transformants formed by HFIR had only a part of the homologous anchor integrated. If the homologous recombination step of the HFIR events was initiated mostly from a paranemic joint (see above), then the result indicates that conversion of a paranemic to a plectonemic joint can occur at any place in the homologous region (Fig. 4) and not preferentially when the paranemic joint extends to the end of the homologous region. The data of Fig. 4 support the view that in the large majority of HFIR events homologous recombination in the anchor is the first step (de Vries & Wackernagel, 2002), fixing heterologous DNA to recipient DNA, which increases its chance for illegitimate recombination outside of the homologous region. Only about 0.01% of the paranemic joints elicit double illegitimate recombination events (Hülter & Wackernagel, 2008).

While invasion of one donor DNA end followed by heteroduplex extension through branch migration as proposed for *S. pneumoniae* transformation (Lacks, 1988; Pasta & Sicard, 1996) could account for the short track integrations as seen in this study, it appears less likely that such a mechanism would effect the integration of segments of several 10 000 nt, and particularly of DNA containing large insertions or deletions, as these constitute blocks to heteroduplex extension (Pasta & Sicard, 1996). More probably, two separate invasions of the 3' and 5' ends of the molecule followed by ligation and segregation through replication could integrate large fragments or fragments with insertions or deletions. This proposed two-end-integration mechanism requires that homologous 3' and 5' ends can invade recipient DNA during transformation, which was established for *S. pneumoniae* (Pasta & Sicard, 1999) and *A. baylyi* (de Vries & Wackernagel, 2002). Further evidence for such a two-end-integration model, which may also apply to *B. subtilis*, *Neisseria gonorrhoeae* and *P. stutzeri*, has been discussed by Kickstein *et al.* (2007). The ratio of events with one invading end (short tracks) and two ends (long segment integration, integration of deletions and insertions) during transformation may depend on the organism studied (e.g. whether the polarity of strand invasion is biased or not), the length of the taken-up DNA, and the genetic marker employed (point mutation vs segment mutation).

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