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Thematic Issue on Horizontal Gene Transfer

Influence of flanking homology and insert size on the transformation frequency of *Acinetobacter baylyi* BD413

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RecA-mediated recombination requires regions of homology between donor and recipient DNA for successful integration. This paper investigates the effect of the relationship between the length of gene-sized inserts (434, 733, 2228 and 2400 bp) and flanking sequence homology (100 – *ca.* 11 000 bp) on transformation frequency in *Acinetobacter baylyi* strain BD413. Both insert size and size of the homologous region were varied, which improves on previous studies that kept insert size constant and varied only the homologous flank size. Transfer frequency of a non-homologous single small gene for gentamicin resistance (*aac*(3)I; 773 bp) was increased 18-fold when flanking homology was changed from about 2000 bp to 8000 bp, but was reduced 234-fold when two genes were inserted (*npt*II-*gfp*; 2400 bp) between similar homologous regions. To investigate the effect of smaller regions of flanking homology (100 – 2000 bp), a partial *npt*II-*gfp* deletion (434 bp) was restored. This confirmed that a minimum of 500 bp on each flank was required for transformation to be affected by flanking homology. The data obtained allowed development of a multiple regression equation to predict transformation frequency from homology, insert size and total fragment size for gene insertions. We also show that the ratio of flanking homology to insert size and not the total size of donor DNA is the most important variable determining transformation frequency. The equation developed was consistent with results previously reported by others, and so will be useful when using *A. baylyi* as a model for gene transfer by transformation in the laboratory, environment and for biosafety.

Keywords: *Acinetobacter baylyi* BD413 / natural transformation / predicting transfer frequency / gene transfer

INTRODUCTION

Natural transformation is the uptake and subsequent integration of exogenous DNA by competent bacteria (Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005). Recombination is mediated by RecA, and requires sequence homology between incoming donor DNA and sites in the bacterial genome (Dasgupta and Radding, 1982). Integration is essential for the expression of DNA in transformed cells. The amount of homology needed for recombination in well studied bacteria such as *Escherichia coli* (Watt et al., 1985), *Ralstonia solanacearum* (Bertolla et al., 1997) and *Bacillus subtilis* (Khasanov et al., 1992) is about 20–70 bp, with increasing transformation frequency as homology increases.

Bacteria in the genus *Acinetobacter* are common in many natural environments and *Acinetobacter baylyi* shows particularly high natural transformation frequencies (*ca.* 10^{-3} transformants per cell; Juni, 1972; Lorenz and Wackernagel, 1994; Vaneechoutte et al., 2006). Many studies using this organism have shown that sequence homology of regions flanking the DNA to be transferred affect transformation frequency. For example, recombination was detected with 183 bp homology (de Vries and Wackernagel, 2002) but there are no published data for smaller regions. Gerischer and Ornston (2001) showed that transfer frequencies of two point mutations fell as distance increased from 2 base pairs to 10.5 kb. Transformation is more efficient with homology on both sides of a sequence, but integration is also possible with one-sided homology, but at reduced frequency (de Vries and Wackernagel, 2002). Sequence identity also affects transformation efficiency: for example, a decrease from 100% to 90% sequence similarity resulted in a 40-fold decrease (Majewski et al., 2000; Shen and Huang, 1986). In addition to a requirement for sufficient homology, Palmen and Hellingwerf (1997) also highlight the importance of intra-cellular nucleases affecting transformation. They have shown that for *A. baylyi* approximately 500 bp of the incoming DNA is degraded during transfer of a point

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mutation. Thus there is a body of literature indicating the importance of the length of flanking homology in relation to transformation frequency, but no systematic study of this effect in *Acinetobacter* in relation to insert size.

Arber (2000) states that a major force in generating diversity is DNA acquisition. Thus the ability to exchange genes horizontally, even at low frequencies, binds all organisms together evolutionarily. Plant DNA is expected to be present in large amounts and ubiquitously in the environment, and Koonin et al. (2001) have proposed that the uptake of this DNA by bacteria would be a significant evolutionary force. Since *A. baylyi* is naturally competent and transformation proficient, it is a good model organism for studying gene transfer between plants and bacteria in nature. The size of the insert relative to the amount of homology required for the integration of non-homologous DNA is of relevance both to this evolutionary process and also to the debate on genetically modified plants, as it is a likely limiting factor for horizontal gene flow of whole plant genes or transgenes into environmental populations of bacteria. The aim of this work was to determine the amount of flanking homology required for efficient integration of whole genes into *A. baylyi*, and to determine the relationship between the insert size, the amount of flanking sequence homology and transformation frequency.

RESULTS AND DISCUSSION

Constructs used in experiments

Two types of genetic construct have been used in these experiments as donor DNA. The first type of construct used partial *A. baylyi* 16S rRNA gene flanks to act as homologous regions around firstly a gentamicin resistance gene (*Aac*(3)I) and secondly two adjacent genes, encoding green fluorescent protein (*gfp*) and kanamycin resistance (*npt*II). The gentamicin construct (called BC3) acted as donor to provide a 773 bp insert for transfer and the *npt*II-*gfp* donor insert (called PC1) was either 2228 bp or 2400 bp depending on the promoter used (SP6 and psbA respectively). In both cases the 16S rRNA gene homology allowed transfer into one or more of the seven 16S rRNA genes in the wild type *A. baylyi* recipient genome (Gralton et al., 1997). Using 16S rRNA genes in this way as sites for transformation is neither lethal (Strätz et al., 1996) nor does it reduce growth rate (Asai et al., 1999). In experiments using lysates of *A. baylyi* carrying these constructs as donor DNA, the genes were inserted into the genome of the donors *via* the 16S flanks of the constructs. The second type of construct was a recipient containing the *npt*II-*gfp* genes with a 434 bp deletion at the gene boundary, either when chromosomally integrated into the *A. baylyi* genome (BC1) or when carried on a plasmid (BC2). These recipients were used in marker rescue experiments with various lengths of donor DNA, obtained from the complete *npt*II-*gfp* gene construct by appropriately designed PCR primers. These constructs allowed transformation experiments to be done with various lengths of flanking homology for three different gene sized inserts, namely 2228–2400 bp, 773 bp and 434 bp.

Whilst making our constructs in the pGEM T easy plasmid, a pUC19 derived vector, we found that they were taken up during natural transformation and stably maintained by BD413, confirming a recent report (Gralton et al., 1997) showing that ColE1 based plasmids can replicate autonomously in BD413. For this reason we have used linear donor DNA in all the experiments reported here, either as PCR product, linearized plasmid or genomic DNA. This means that recombination always involved an exchange of DNA. If we had used entire or intact plasmids, this would have made interpretation more difficult, as these could become integrated by the Campbell-type integration process *via* their shared *npt*II-*gfp* homology.

Transformation experiments performed with lysates

Transfer frequency of rifampicin resistance (a point mutation) using *A. baylyi* lysates as donor DNA and wild-type *A. baylyi* as recipient was $9.33 \pm 1.2 \times 10^{-3}$ (Tab. 1). Transformation frequencies from lysates of our constructs with insert sizes 773–2400 bp inserted into the *A. baylyi* genome as donor, with wild-type *A. baylyi* as recipient ranged from $2.34–5.15 \times 10^{-3}$ transformants per recipient (Tab. 1) (N.B. these units are used for all subsequent transformation frequencies given here). Thus there was no significant difference in transformation frequency between the recombination of a point mutation in a coding gene and insertion of the 773–2400 bp gene constructs used, when the DNA sequence was presented as a cell lysate, which is when the homologous flanks were very large (at least greater than 3000 bp each; Davidoff-Abelson and Dubnau, 1973). Although the chromosome of *A. baylyi* BD413 has seven copies of the 16S rRNA gene (Gralton et al., 1997), the maximum recombination frequency obtained using the 16S rRNA sequence flanks in the donor construct was not significantly different from the maximum obtained for auxotrophic markers or the rifampicin resistance phenotype (around $10^{-2}–10^{-3}$; Juni and Janik, 1969; Nielsen et al., 1997; Palmen et al., 1993).

Effect of total homology <2000 bp on transformation frequency

Marker rescue experiments repaired the 434 bp deletion using PCR products with flanking sequences around the

Table 1. Transformation of unmodified wild type *Acinetobacter baylyi* (strain BD413) with donor DNA of different flanking homology.

Donor DNA[1]		No. of transformants[2] (mL^{-1})	Transformation frequency[2,3] (transformants/ recipient cell)	Insert size (bp)	Flanking sequence homology[4] (bp)	Total homology[5] (bp)
BD413 lysate	Rif resistant BD413	$6.95 \pm 1.21 \times 10^5$	$9.33 \pm 1.20 \times 10^{-3}$	Point mutation	*	> 6000
	PC1-SP6 in genome	$7.16 \pm 241 \times 10^5$	$2.34 \pm 0.60 \times 10^{-3}$	2228	*	> 6000
	PC1-psbA in genome	$8.89 \pm 2.63 \times 10^5$	$3.25 \pm 0.88 \times 10^{-3}$	2400	*	> 6000
	BC3 in genome	$1.10 \pm 0.15 \times 10^6$	$5.15 \pm 1.60 \times 10^{-3}$	773	*	> 6000
PCR product	PC1-SP6	12.5 ± 7.5	$1.38 \pm 0.01 \times 10^{-8}$	2228	246 & 196	442
	PC1-psbA	8.3 ± 1.7	$2.10 \pm 0.74 \times 10^{-8}$	2400	246 & 196	442
	BC3	6.43 ± 5.11	$7.50 \pm 2.59 \times 10^{-9}$	773	246 & 196	442
Linear plasmid	PC1-SP6	4.0 ± 3.06	$2.29 \pm 1.23 \times 10^{-8}$	2228	246 & 196	442

[1] All donor DNA except the Rif resistant lysate contained 16S rRNA homologous flanks, see Materials and Methods for details of constructs.
[2] Results are mean of 3 to 10 replicates ± SE of mean.
[3] MSD = 0.972 $\log_{10}$ units at $P = 0.05$ (and 1.169 at $P = 0.01$), this shows that the transformation frequencies for (i) lysates and (ii) linear donor DNA (PCR product and linear plasmid) are significantly different from each other but not within these groups.
[4] Numbers indicate left and right lengths of flanking sequences; * indicates that the lengths of these sequences could not be determined as the donor was lysate DNA (see footnote[5]).
[5] Maximum total donor fragment size for lysates taken up by BD413 was assumed to be >6000 bp (Davidoff-Abelson and Dubnau, 1973).

434 bp deletion site from intact *npt*II-*gfp* genes amplified from construct PC2. These showed the effect of the length of flanking homology, using 24 combinations of flanking sequence with flanks between 50 bp and 1100 bp. The results (Fig. 1A) showed a random cloud of transfer frequencies (geometric mean = 2.01×10^{-7}) when one or both regions of flanking homology were less than 500 bp. These values were not significantly different from each other, were not linearly correlated, and the slope of line of best fit was not significantly different from zero ($r = -0.31$; $R^2 = 9.5\%$; $P = 0.119$), which confirms the randomness of these values. This low frequency of transformation cannot be due to contamination of the PCR product with genomic DNA, because control experiments carried out with genomic DNA diluted to the concentrations expected in the PCR products gave either no transformants or frequencies very much lower than those observed with >500 bp flanking homology. However, when both flanks were >500 bp in length, the transfer frequencies increased linearly ($r = 0.986$; $P = 0.002$), with the total homology on the semi-logarithmic plot presented (Fig. 1A). This indicates the dependence of transfer frequency on homology size for this 434 bp insert when homology is above 500 bp on both flanks.

Note that in Figure 1A the recipients carried the deleted genes both chromosomally (from construct BC1) and on a plasmid (BC2). These results are presented together, as there was no significant difference between the transfer frequencies for these constructs (BC1 = 4.4×10^{-5}; BC2 = 4.0×10^{-5}; $P = 0.856$), despite a potential 10-fold difference in target copy number, with seven copies of the 16S rRNA gene in *Acinetobacter* sp. BD413 (Gralton et al., 1997), and 10–50 copies of the plasmid per cell (West et al., 1994).

Notably, our data support the model proposed by Pifer and Smith (1985) for *Haemophilus influenzae*, and confirmed for *A. baylyi* BD413 (Palmen and Hellingwerf, 1997), in which processing of donor DNA results in a requirement for a minimum sized flanking sequence for efficient recombination, due to exonuclease degradation of the incoming single-stranded DNA before recombination can be started. Palmen and Hellingwerf (1997) showed that this minimum flanking size was 500 bp for *A. baylyi* BD413 when repairing a point mutation, and our results show that this minimum flank size remains 500 bp for a much larger 434 bp fragment.

Our results differ from Palmen and Hellingwerf (1997) at low transformation frequencies. We have shown that, within the limits of variability, transformation frequency was constant at about 2.0×10^{-7} (range = 2.5×10^{-8}–1.0×10^{-6}; n = 19), whereas Palmen and Hellingwerf (1997) supposed that a semilogarithmic trend (n = 3) was observed even at low frequencies. Thus our data suggest that transformation reaches a minimum frequency that is not decreased further by decreasing the size of homologous flanks down to 50 bp upstream and downstream of the insert DNA. This is the shortest amount of flanking sequence shown to be active in

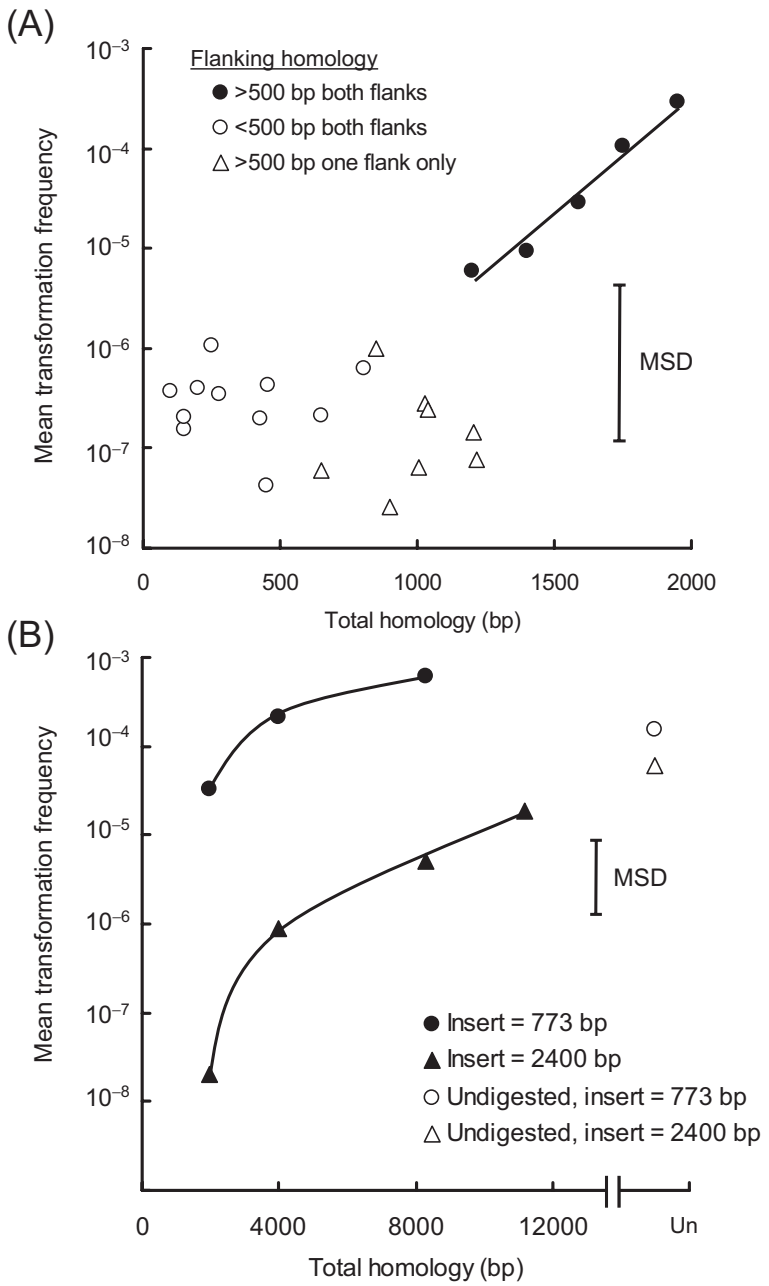

Figure 1. Effect of flanking homology on transformation frequency in *A. baylyi* BD413. Transformation frequency is expressed as transformants per recipient cell. (A) Experiments with PCR products (n = 5–12), using the marker rescue approach with flanking homologies from 50–>850 bp on each side (see Tab. 5) of the donor DNA. The recipient contained deletion inactivated *npt*II and *gfp* genes (434 bp deletion), in constructs BC1 (integrated into the *A. baylyi* genome) and BC2 (carried on plasmid). These strains were transformed with donor DNA from PC2 PCR products, intact *gfp* and *nptII* genes or portions thereof. (B) Cell lysate experiments (n = 3–12). The wild type recipient was transformed with genomic donor DNA from two strains of *A. baylyi* carrying either construct BC3 (*aac*3(I); 773 bp) or PC1 (*npt*II-*gfp*; 2400 bp) inserted into a 16S rRNA gene. The genomic DNA was digested with restriction enzymes to produce flanking homology sequences of 1974 to 11 195 bp and the recipient was wild type BD413. Un = control values using undigested donor DNA, plotted separately on the right because the total homology is large but cannot be accurately estimated. MSD = minimum significant difference from the Tukey-Kramer test after analysis of variance (see Materials and Methods for details).

Acinetobacter to date, and is comparable to that seen in other species (Bertolla et al., 1997; de Vries and Wackernagel, 2002; Khasanov et al., 1992).

Transformation frequencies using PCR products of the 773–2400 bp gene inserts with small flanking sequences (246 and 196 bp) as donor DNA were also low (7.5×10^{-9}–2.1×10^{-8}; Tab. 1). These values are significantly lower ($P < 0.001$) than those obtained with the 434 bp insert discussed above. These results indicate that there might be a minimum level of flanking homology beyond which transformation frequency does not decline further. So at saturating donor DNA concentrations some molecules will always escape exonuclease degradation during DNA entry into *A. baylyi* BD413, but the proportion that escape depends on the length of the homologous flanks around the insert.

It is interesting to note that ScaI cuts the plasmid backbone at position 1876 of the pGEM plasmid backbone thus providing 1820 bp and 1180 bp of non-homologous plasmid sequence on the two sides of the insert in addition to the 246 bp and 196 bp of homologous flanking sequence. The transformation frequency into wild type recipients of this construct was 2.29×10^{-8} (Tab. 1). So there was no significant increase in the transformation frequency by the addition of non-homologous flanking sequences exceeding the 500 bp which on average is removed during DNA processing on entry into the cell (see Palmen and Hellingwerf, 1997; and above).

Effect of total homology >2000 bp on transformation frequency

To explore the effect of homology on transformation frequency of larger gene inserts further, the PCR products of the intact gentamycin (*Aac*(3)I) and *nptII-gfp* genes were integrated into the *A. baylyi* genome using the 16S rRNA flanking homologous sequences to generate donor strains carrying PC1 and BC3 inserts. Genomic DNA of *A. baylyi* PC1-psbA (*nptII-gfp*; insert size 2400 bp) and BC3 (*aac*3(I); insert size 773 bp) was digested with restriction enzymes to produce fragments containing the insert with total flanking sequence homology ranging from 1974 to 11 195 bp. The upstream and downstream flanking homology varied between 641 bp and 6976 bp for these fragments. These restriction digestion products were used as donors to transform wild-type *A. baylyi* BD413. Undigested genomic DNA from *A. baylyi* PC1 and *A. baylyi* BC3 was used as controls to represent the maximal flanking sequence available. These data show that for both insert sizes transformation frequency increased with increasing total homology (Fig. 1B). Furthermore, the frequency for the smaller insert increased less than the larger insert, and was over 1000-fold higher with 1974 bp of homology. All the frequencies with the 2400 bp insert were significantly different from the control frequencies using undigested genomic DNA, apart from that obtained with largest total homology (1974 bp, $P < 0.001$; 3991 bp, $P < 0.001$; 8296 bp, $P = 0.006$; 11 195 bp, $P = 0.133$). For the smaller construct, insert size 773 bp, transformation frequency was not significantly different from the control at any homology level.

These results show that for these larger inserts transformation frequency is dependent on total homology when flanking homology is greater than 500 bp on each side of the insert. It is also clear that the larger insert showed lower frequencies for similar lengths of homology than the smaller insert, and so transfer frequency is also dependent on insert size. The curved shape of the relationships between frequency and total homology for both insert sizes is similar to the shape predicted by Pifer and Smith (1985) for their competition model for the mechanism of transformation discussed earlier for large transforming DNA fragments. Interestingly, the linear relationship noted (Fig. 1A) for the 434 bp insert with less flanking homology is equivalent to the linear relationship predicted for small DNA fragments from the Pifer and Smith (1985) model.

Relating transformation frequency to total homology and insert size

Multiple regression analysis was carried out on all data obtained from the experiments with constructs carrying insert sizes of 434 bp, 773 bp, 2228 bp and 2400 bp, and total flanking homology regions ranging from 100 to 11 195 bp (n = 37). The aim of this analysis was to determine which variables affected transformation frequency most, and to obtain a useful prediction equation to help other studies. The data for undigested DNA were not included in these regression analyses, as the size of the homologous flanking sequence could only be estimated. The best regression equation used the ratio of total homology to insert size, minimum homology and total fragment size as variables to predict transformation frequency. We did look at other variables (relative size and orientation of the flanking sequences, $\log_{10}$ transformations of all the predictor variables) but these did not explain any more variability in the data. Regression with these variables showed that 60% of the variation in $\log_{10}$ transformation frequency observed could be explained by the ratio of total homology to insert size and that 67% of the variation was accounted for by all three variables, but no more than 60% of the variation was explained by the ratio and either of the other two variables. In all regressions with all combinations of variables, the ratio was always the first variable selected by stepwise regression, and so can be considered the most important variable affecting transformation frequency. Furthermore, bivariate

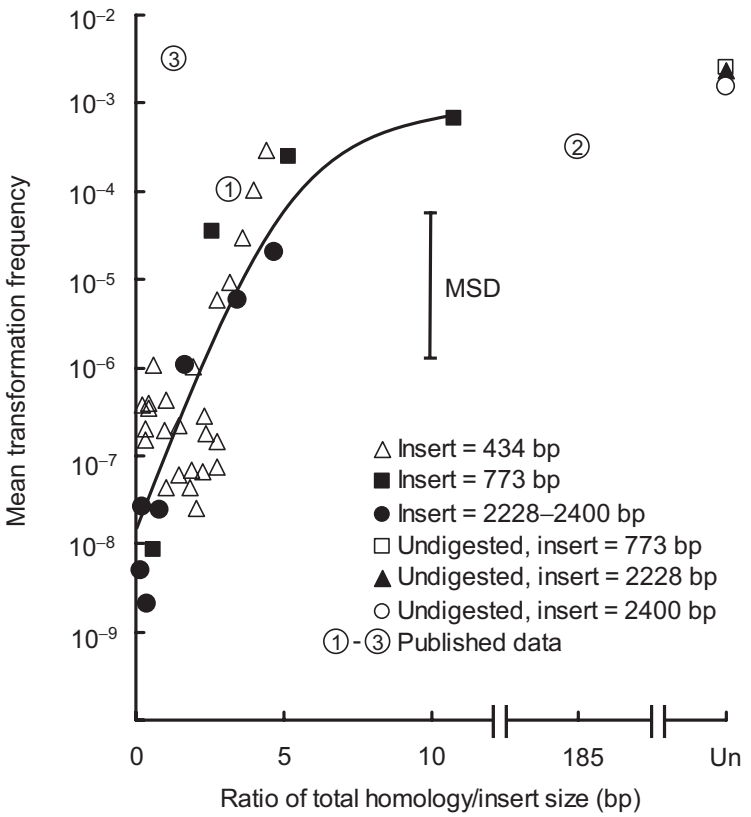

Figure 2. Effect of the ratio of total homology to insert size on transformation frequency in *A. baylyi* BD413. The ratio of total homology to insert size was plotted for all data used in this study (overall means from all experiments, N = 39, n = 2–22). Recipients were (i) wild type *A. baylyi* for 773 bp, 2228 bp and 2400 bp inserts flanked by 16S rRNA sequences and (ii) *A. baylyi* carrying inactivated *gfp* and *nptII* genes BC1 (chromosomally integrated) and BC2 (carried on plasmid). The circled numbers refer to data from published studies: 1 = Gebhard and Smalla (1998); 2 = de Vries and Wackernagel (2002); 3 = Strätz et al. (1996). Transformation efficiency is expressed as transformants per recipient cell. Un = control values using undigested donor DNA, plotted separately on the right because the total homology is large but cannot be accurately estimated. MSD = minimum significant difference from the Tukey-Kramer test after analysis of variance (see Materials and Methods for details).

regressions between transformation frequency and total homology, maximum and minimum homology and fragment size never accounted for more than 31% of the variation in transformation frequency (range = 27–31%).

The best equation from this analysis is as follows:

$$\mathrm{Log}_{10}Tf = -6.73 + 0.343\,R_{hi} + 0.00196\,H_{min} - 0.000567\,F_{tot}$$

where: Tf = mean transformation frequency;

R_{hi} = total homology divided by insert size;

H_{min} = size of minimum homologous flanking sequence;

F_{tot} = total fragment size.

This analysis shows that decreasing the amount of flanking homology affected transformation frequency depending on insert size (Fig. 2). Also, this equation will allow predictions of transformation frequency to be made for a given construct with sizes of insert and flanking homology falling within the range of our study, when transformed into *A. baylyi* BD413 under the conditions tested. This will be useful information for designing constructs for studies involving recombination, and for predicting the likely frequency of horizontal gene transfer.

Few studies reported in the literature give values for both insert size and the length of flanking homologous regions. Despite this, when our equation was used to predict transformation frequencies from the literature, data from two studies (de Vries and Wackernagel, 2002; Gebhard and Smalla, 1998) fall well within the spread of our data (Fig. 2). The result from a third study (Strätz et al., 1996) is 10^4 fold higher than we predict from our equation. In this work Strätz et al. (1996) obtained a frequency of 3×10^{-3} transformants per recipient of a single marker

gene, *ApH*3, into the *A. baylyi* BD413 chromosome. However, donor DNA was presented as purified plasmid in the pDirect cloning vector (Clontech), which is a pUC19 derivative. Although Strätz et al. (1996) confirmed chromosomal integration had occurred, no check was reported to establish the absence of the plasmid. So, as discussed earlier, it is possible that many of their transformants may also have contained plasmid-borne copies of the construct, which would have enhanced the apparent transformation frequency. Thus their recombination frequencies using this vector may be artificially high.

Significance of this research

By deriving an equation that explains 67% of the variability in transformation frequency caused by changes in the ratio of total homology to insert size, minimum homology and total fragment size, we have extended previous work on the factors controlling transformation frequency in bacteria (*e.g.*, de Vries and Wackernagel, 2002; Palmen and Hellingwerf, 1997; Pifer and Smith, 1985). Furthermore, the transformation model proposed by Pifer and Smith (1985) and Palmen and Hellingwerf (1997) is confirmed for gene sized inserts by our results.

An issue we have not addressed so far is the presence of hot spots in bacteria, where recombination frequencies are higher (Stahl et al., 1975). However, given the similar transformation frequencies obtained with integration of our constructs into the 16S rRNA genes and the restoration of rifampicin resistance, there was no evidence of the 16S rRNA genes acting as hot spots (or cold spots). In addition, we included published data in our work to ensure its robustness, and found that our data are consistent with that of others (Fig. 2); thus we believe the equation has general applicability for *Acinetobacter* BD413, within the limits of homology and insert size used here.

Finding a clear relationship between insert size and length of flanking sequence with transformation frequency has clear implications for understanding horizontal gene transfer by natural transformation of bacteria, both in the laboratory and more widely in field studies. Recently, it was predicted that over the 70 million hectares planted with transgenic crops, we might expect over 10^{18} bacteria to acquire plant transgenes based on a transformation frequency of 10^{-17} (Heinemann and Traavik, 2004). Given that direct detection of transformation in the field at such low frequencies is highly problematic, incorporating all the variables into estimates of baseline transformation frequencies is important in making these kinds of global estimates. Our prediction equation is based on use of saturating DNA concentrations, which are unlikely to occur in the field, so our equation is currently restricted to laboratory use. However, similar approaches to ours could be used to take into account insert size and length of homology in more comprehensive models, especially relevant to the transfer of plant transgenes to bacteria in the environment (Nielsen and Townsend, 2004; Pettersen et al., 2005).

Conclusion

This research shows that in *A. baylyi* a minimum of 500 bp on each side of gene sized inserts was required for transformation to be affected by flanking homology and confirms that whole genes behave like point mutations in this respect. A multiple regression equation was developed from all the data, which predicted transformation frequency from homology, insert size and total fragment size for gene insertions. Our experiments also show that the ratio of flanking homology to insert size and not the total size of donor DNA was the most important variable determining transformation frequency. The equation developed will provide a useful base line when using *A. baylyi* as a model organism for gene transfer of whole genes by transformation in the laboratory, environment and for biosafety research. Our work points the way to the sort of experiments that should be considered for establishing prediction models that can be used to guide field studies and facilitate better estimation of the environmental transfer of transgenes from plants to bacteria.

MATERIALS AND METHODS

Bacterial strains

E. coli strains XL1 Blue (Stratagene, Cedar Creek, Texas, USA), Top10 (Invitrogen, Carlsbad, California, USA), and JM109 (Promega, Madison, Wisconsin, USA) were used for cloning the constructs. These strains are *rec*A$^-$ to prevent integration of the constructs into the *E. coli* 16S rRNA chromosomal genes, *via* homology provided by the *Acinetobacter* 16S rRNA gene flanking sequences. The non-methylating *E. coli* strain SSC-110 (Stratagene) was used for preparing plasmids for *Cla*I digestion. *A. baylyi* strain BD413 (Vaneechoutte et al., 2006) was used as the recipient in transformation experiments. A strain of rif-resistant BD413 was obtained by spontaneous mutation on LB + rifampicin (100 $\mu g.L^{-1}$) medium. All strains were grown in LB medium (10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ NaCl), and on LB agar plates (1.5% agar). *E. coli* cultures were incubated at 37 °C. *A. baylyi* BD413 was grown at 30 °C. Other antibiotics used in media were as follows, kanamycin (50 $\mu g.L^{-1}$), gentamicin (15 $\mu g.L^{-1}$) and carbenicillin (50 $\mu g.L^{-1}$).

Table 2. PCR primers used for making constructs and transformant identification.

Primer	Sequence 5' to 3' (restriction sites underlined)	Restriction site
nptIIA	ATGCGGATCCCTTTGACGTTGGAGTCCA	*Bam*HI
nptIIB	CACGATCGATCTCAAGGATCTTACCGCT	*Cla*I
nptIIC	ATCAGTACGCGT GGATCCCTTTGACGTTGGA	*Mlu*I; *Bam*HI
nptIID	CAGTGAATTCCTTTGACGTTGGAGTCC	*Eco*RI
GFP1	GTGCGTCGACCTCTACTAGTGATCTCAATGAATATTGGTTGAC	*Sal*I
GFP2	CGCTCCGCTTATTTGTATAGTTCATCCATGC	*Aci*I
GFP3	ACTGGTCGACGCTATTTAGGTGACACTATA	SalI
GFP4	AGCTGAATTCGATCTCAATGAATATTGGTTGA	*Eco*RI
GFP5	ACTGTGAATTCGACTCCTACGGGAGGCAGCA	*Eco*RI
16S1	ACGCTGAATTCGCTATTAGGTGACACTATAG	*Eco*RI
16S2	CGATGTCGACGCGGTGTGTACAAGGC	*Sal*I
GENT1	ACGTGGATCCAGACTCGAATTGACATAA	*Bam*HI
GENT2	TGCAGGATCCCGAATTGTTAGGTGGC	*Bam*HI
GENT3	TCAGGCGCGCAGCTCGAATTGACATAAG	*Bss*HII
GENT4	ATCTGGCCCTAACGGCCTGAATTGTTAGGTGGC	*Sfi*I

Molecular methods

PCR was carried out using the MJ Research DNA engine (Genetic Research Instrumentation, Braintree, Essex, UK), using standard conditions. *Pfu* DNA polymerase (Promega) was used to amplify genes for cloning, and *Taq* DNA polymerase (Promega) was used for screening by colony PCR. PCR amplification was carried out over 30 cycles, with 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and 1 min extension per kb product with *Taq* DNA polymerase or 2 min per kb product with *Pfu* DNA polymerase at 74 °C. PCR primers are listed in Table 2. PCR products were purified by using the Qiagen PCR purification kit. Plasmid minipreps were carried out on 1.5 mL overnight cultures using the GenElute plasmid miniprep kit (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK). Restriction digests were performed using enzymes from Promega and New England Biolabs (Ipswich, Massachusetts, USA).

Production of donor DNA constructs

The *npt*II gene and its promoter (derived from the Tn5 transposon) were PCR-amplified from the pCRII cloning vector (Invitrogen). The *gfp* gene and *psb*A promoter were PCR-amplified from the pUT*gfp-lux* plasmid (Timms-Wilson and Bailey, 2001; Tab. 2), which contains a red-shifted *gfp* variant with maximum expression when excited with blue light (471 nm) (Heim et al., 1994). The *psb*A promoter originates from the chloroplast of *Amaranthus hybridus* (Elhai, 1993). The *acc*(3)I gentamicin resistance gene was PCR-amplified from pUCP24 (West et al., 1994; Tab. 2). Cloning was carried out using pGEM T Easy vector (Promega), and constructs for expression in *A. baylyi* BD413 were produced in the broad host range plasmid pUCP24 (West et al., 1994).

The constructs described in this paper were produced to evaluate the movement of transgenes from transgenic plants to soil bacteria. They were designed using 100% homologous, two-sided, flanking sequences based on amounts of homology required for efficient integration (Bertolla et al., 1997; de Vries and Wackernagel, 2002; Watt et al., 1985). Plasmid structures are illustrated in Figure 3. Three plasmid constructs were produced to provide donor sequences. Two of these pGEM-PC1 and pGEM-PC2 included either the psbA or SP6 promoters. Only the *psb*A constructs are shown (Fig. 3) and are referred to as PC1-psbA (Fig. 3D) and PC2-psbA (Fig. 3B) respectively. Variant constructs with a SP6 promoter (replacing the *psb*A promoter), identical apart from this sequence, were also constructed. The third plasmid is pGEM-BC3, referred to as BC3 (Fig. 3E). Cloned sequences of both BC3 and PC1 plasmid constructs were flanked by the same conserved 442 bp 16S sequence. These plasmids were constructed as follows. The *npt*II gene was PCR-amplified as a 1459 bp fragment, from nucleotide 838–2299 of pCRII, using primers nptIIA and nptIIB (Tab. 2). After purification, the *npt*II fragment was ligated into pGEM T Easy vector, transformed into *E. coli*

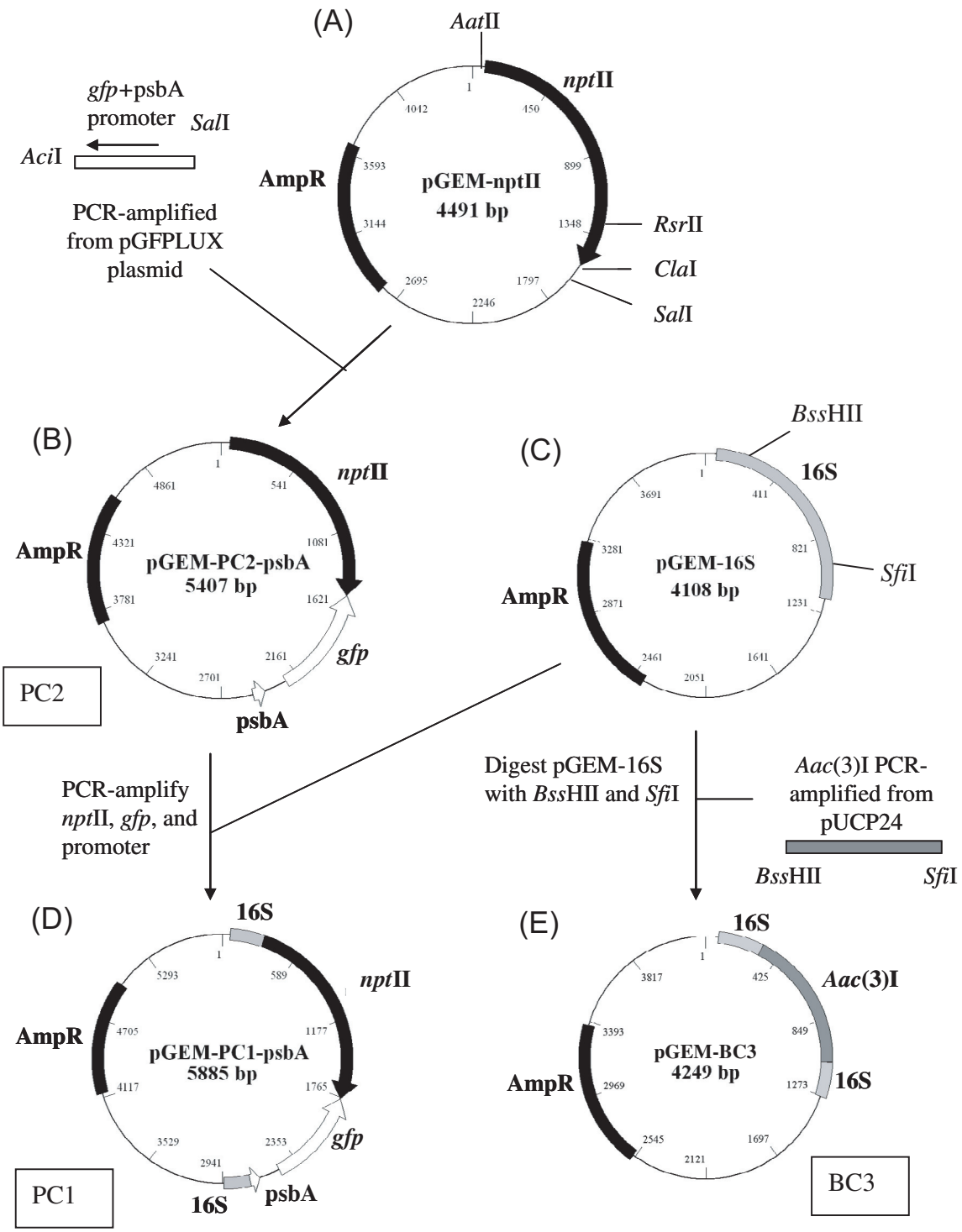

Figure 3. Donor DNA *gfp* expression-constructs; plasmids PC1-psbA, PC2-psbA and BC3. Plasmids with *gfp* expression-constructs PC1-SP6 and PC2-SP6 were also made and are 172 bp smaller.

XL1 blue cells (Stratagene) and plated on LB plates supplemented with 50 μg.mL^{-1} kanamycin. The orientation of the *npt*II insert was determined by restriction digestion with *Aat*II and *Rsr*II. A plasmid clone, pGEM-*npt*II, (Fig. 3A) was selected with *npt*II oriented in the 5' to 3' direction.

To produce plasmid construct PC2 (pGEM-PC2-psbA in Fig. 3B), *gfp* driven by the *psb*A promoter was PCR-amplified from pUT*gfp-lux* plasmid using primers GFP1 and GFP2 (Tab. 2). The PCR product was purified, digested with *Aci*I (to produce compatible sticky ends to *Cla*I) and *Sal*I, and ligated into the pGEM-*npt*II plasmid digested with *Cla*I and *Sal*I. The ligation was transformed into *E. coli* JM109 (Promega) using kanamycin selection. GFP fluorescence was monitored under blue light, using a GRI dark reader transilluminator (400–500 nm, maximum output at 450 nm). To confirm insertion of *gfp* into the plasmid, fluorescent colonies were screened by colony PCR using primers GFP1 and nptIIA (Tab. 2). Plasmids were sequenced to check the *gfp* gene orientation and the presence of all cassette components in the construct. In some clones, the psbA promoter was lost spontaneously and *gfp* expression was controlled by the SP6 promoter present in the pGEM vector. These plasmids were termed pGEM-PC2-SP6.

Plasmid PC1 (pGEM-PC1-psbA in Fig. 3D) comprised *npt*II and *gfp* genes flanked by *A. baylyi* BD413 16S rRNA gene sequences. The *Acinetobacter* sequences were selected to have highly conserved regions at the outer edges (NCBI Accession number X89709, upstream flank 305–546 and downstream flank 1176–1373 numbered from the start of this sequence). PCR primers were designed to amplify the *Acinetobacter* 16S RNA gene from the region 305–1373 (1069 bp). This region was amplified from BD413 genomic DNA using primers 16S1 and 16S2 (Tab. 2). Adenosine residues were added to the resulting fragment before ligation into the cloning site of pGEM T Easy vector. Ligation mixtures were transformed into Top10 cells (Promega) and transformants were selected on ampicillin. The presence of the 16S fragment was determined by colony PCR using primers 16S1 and 16S2 (Tab. 2), and the orientation was determined by restriction digestion and sequencing. Clones with the 16S rRNA gene fragment running in the 5' to 3' direction were selected. This plasmid was termed pGEM-16S (Fig. 3C).

Plasmid pGEM-16S (Fig. 3C) was digested with *Sfi*I, and the ends were filled using T4 DNA polymerase before digestion with *Bss*HII to remove the central section of the 16S rRNA gene, and purification by gel extraction. To produce plasmid PC1 (pGEM-PC1-psbA in Fig. 3D), the *npt*II-*gfp* cassette was PCR-amplified from plasmids pGEM-PC2-SP6 and pGEM-PC2-psbA, using primers nptIIC and GFP1, and nptIIC and GFP3 respectively (Tab. 2). The PCR products were purified and digested with *Mlu*I, which creates compatible ends to *Bss*HII, before ligation with the pre-digested pGEM-16S plasmid (Fig. 3C), and transformation into *E. coli* JM109. Transformants were selected for kanamycin resistance (50 μg.mL^{-1}) and GFP fluorescence, and confirmed by PCR, and restriction digestion. The resulting plasmids were termed pGEM-PC1-SP6 and pGEM-PC1-psbA (Fig. 3D). To produce plasmid BC3, the *acc*(3)I gene with promoter was amplified from pUCP24 using primers GENT3 and GENT4 (Tab. 2). The PCR product was digested with *Bss*HII and *Sfi*I, and ligated with pGEM-16S (Fig. 3C) pre-digested with the same enzymes, before transformation into *E. coli* JM109. Transformants were selected on gentamicin (15 μg.mL^{-1}), and termed plasmid construct BC3 (pGEM-BC3 in Fig. 3E). Inserts from PC1 and BC3 were subsequently transformed into the *A. baylyi* genome using the 16S rDNA flanking sequences for recombination into one or more of seven 16S rRNA genes in the *A. baylyi* genome.

Production of recipient DNA constructs

Two plasmid constructs were produced containing inactivated *npt*II and *gfp* genes; sequences from these constructs were subsequently transformed into *A. baylyi* BD413. These plasmid constructs were pGEM-BC2 (Fig. 4C) and pGEM-BC1 (Fig. 4E). Note that the donor plasmid constructs PC1-psbA and PC2-psbA, and recipient plasmid constructs BC1-psbA and BC2-psbA were also produced with the SP6 promoters (*i.e.*, PC1-SP6, *etc.*) but only the *psb*A versions are shown in Figure 4.

The GFP protein is inactivated by the removal of nine amino acids from the C-terminal end (Li et al., 1997), and the nptII protein can be inactivated by the removal of 22 amino acids from the C-terminal end (Beck et al., 1982). As the coding portion of the C-terminal ends were adjacent in plasmid construct PC2, both genes were inactivated by digestion with *Rsr*II and *Bst*BI (Fig. 4A), which removes 150 bp from *npt*II and 100 bp from *gfp*. After digestion, the sticky ends were filled in using T4 DNA polymerase (Promega), the plasmid was religated, and used to transform *E. coli* JM109, to produce plasmid BC2 (pGEM-BC2-psbA in Fig. 4C). Transformed *E. coli* colonies containing plasmid BC2 were no longer kanamycin resistant or fluorescent and were therefore selected using carbenicillin (AmpR) at 50 μg.mL^{-1}. To transfer the BC2 insert into *A. baylyi* BD413 the *npt*II-*gfp* region of plasmid BC2 was PCR-amplified using primers nptIIA and either GFP4 (for BC2-psbA) or GFP5 (for BC2-SP6), and ligated into the broad host range plasmid pUCP24, digested with *Bam*HI and *Eco*RI. The ligation mixture was used to transform *E. coli* JM109 cells and transformants were selected on gentamicin. Subsequently

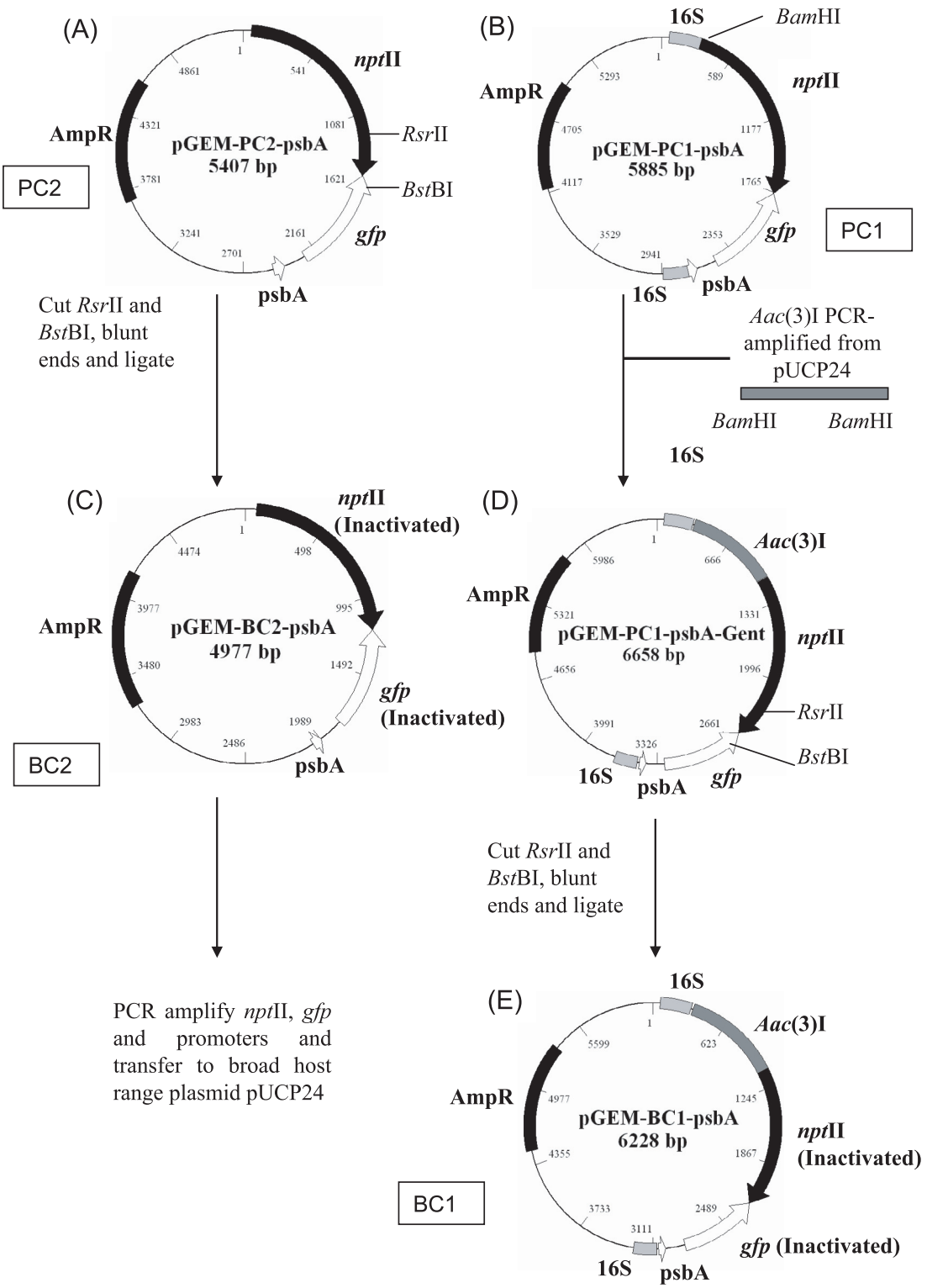

Figure 4. Recipient DNA plasmid constructs BC1 and BC2 with deletion inactivated *npt*II and *gfp* genes. Plasmids pGEM-BC1 and pGEM-BC2 were also produced with *gfp* expression controlled by the SP6 promoter, which results in plasmids that are 172 bp smaller.

Table 3. Restriction enzymes used for genomic DNA digestion.

Restriction enzyme	Sequence homology (bp)		
	Left flank	Right flank	Total
*Bsp*HI	1333	641	1974
*Nsi*I	2551	1440	3991
*Mlu*I	5067	3229	8296
*Eco*RV[1]	6976	4219	11 195

[1] *Eco*RV used for *A. baylyi* BD413 carrying construct PC1 in genome digestion only.

the pUCP24 plasmid carrying the BC2 construct was transformed into *A. baylyi*.

To produce plasmid BC1 the *acc*(3)I gene (conferring resistance to gentamicin; PCR product size 773 bp) was PCR-amplified from pUCP24 using primers GENT1 and GENT2 (Tab. 2) and digested with *Bam*HI. The PCR product was ligated with plasmid PC1 (Fig. 4B) pre-digested with *Bam*HI, to produce pGEM-PC1-psbA-GENT (Fig. 4D). This was digested with *Rsr*II and *Bst*BI as described for plasmid BC2, to produce plasmid construct BC1 (insert size 3170 bp; pGEM-BC1-psbA in Fig. 4E).

*Sca*I linearised plasmid and PCR product (PCR product size 3670 bp) amplified from plasmid construct BC1 using primers 16S1 and 16S2 (Tab. 2) were used to transfer the BC1 insert into the *A. baylyi* BD413 genome, and transformants were selected for gentamicin resistance.

Preparation of donor DNA

Genomic DNA was extracted from *A. baylyi* BD413 containing inserts PC1 and BC3 by CTAB extraction (Juni, 1972). Overnight culture (1.5 mL) was centrifuged at top speed in an Eppendorf 5415C centrifuge for 5 min. The supernatant was removed and the bacteria were resuspended in 500 μL of lysis solution (100 $\mu g.mL^{-1}$ proteinase K, in 0.5% SDS) and incubated at 55 °C for 60 min, followed by addition of 100 μL 5M NaCl, and 80 μL CTAB solution (10% w/v CTAB in 0.7M NaCl). After mixing by inversion the samples were incubated for 15 min at 65 °C, before 680 μL chloroform:isoamyl alcohol (1:25) was added and the tubes shaken gently to form an emulsion, and centrifuged for 5 min. The upper aqueous layer was transferred to a clean tube and the DNA was collected by isopropanol precipitation. Genomic DNA was digested with the restriction enzymes listed in Table 3, which were selected on the basis of their presence in *A. baylyi* BD413 16S rRNA gene, and absence from the inserted marker genes. BC3 genomic DNA was not digested with *Eco*RV due to the presence of an *Eco*RV site within the gentamicin resistance gene. Linear plasmids of pGEM-PC1 and BC3 were prepared by digestion with *Sca*I. PCR products for transformation of inactivated *npt*II-*gfp* construct with varying regions of homology were produced using the primers shown in Table 4, which gave the PCR product sizes given in Table 5. All PCR products were checked for purity by gel electrophoresis. All products were pure, except in cases where product was from constructs with 16S rRNA gene inserts. In these cases two bands were seen, one from the native 16S rRNA gene and one from the construct. Fragments from the constructs were purified by gel extraction and purity confirmed by further gel electrophoresis. Bacterial lysates were produced by resuspending the cells from 1 mL of overnight culture in 500 μL sterile saline citrate (0.15 M NaCl, 0.015 M sodium citrate and 0.05% SDS), and incubating at 60 °C for 1 h with occasional shaking. The resulting lysates were stored at 4 °C until used.

Table 4. PCR primers used to produce donor DNA for transformations.

Primer	Sequence 5' to 3'
50BPF	ATGGCCGCTTTTCTGGATT
50BPR	CTGTCCTTTTACCAGACAACC
101BPF	ACTCGTCGTGATCCATG
100BPR	CGTTCAACTAGCAGACCAT
200BPF	CCGGTCTTGTCGATCAGGATGATCTGGACGAAG
228BPR	GATGGAAACATTCTTGGACAC
400BPF	TGTCACTGAAGCGGGAAG
405BPR	TACCCAGATCATATGAAACAGC
600PBF	GAGAGGCTATTCGGCTATGA
P600BPR	ACTGGAGTTGTCCCAATTC
S600BPR	CTGGAGTTGTCCCAATTCTT
800BPF	GGACAGCAAGCGAACC
986BPF	CAAATTCAGGGCGCAAG
GFP1	GTGCGTCGACCTCTACTAGTGATCTCAATGAATA
	TTGGTTGAC
GFP-3	ACTGGTCGACGCTATTTAGGTGACACTATA
NPTIID	CAGTGAATTCCTTTGACGTTGGAGTCC
16S1	ACGCTGAATTCGCTATTAGGTGACACTATAG
16S2	CGATGTCGACGCGGTGTGTACAAGGC

Transformation experiments

Transformation experiments were carried out as described by Williams et al. (1996). A 25 mm diameter, 0.45 μm pore size nitrocellulose filter was placed on a nutrient LB agar plate with appropriate antibiotic selection: 50 $\mu g.mL^{-1}$ kanamycin in transformations using donor

Table 5. PCR products used as donors for transformation experiments with 434 bp insert constructs.

Left flank (bp)	Right flank (bp)	Total homology (bp)	PCR product (bp)
50	50	100	540
50	101	151	591
50	228	278	718
50	405	455	895
50	600	650	1090
101	100	201	641
101	50	150	590
200	228	428	868
200	50	250	690
400	405	805	1245
400	50	450	890
600	600	1200	1640
600	50	650	1090
600	405	1005	1445
800	600	1400	1840
800	50	850	1290
800	100	900	1340
800	228	1028	1468
800	405	1205	1645
989	600	1589	2029
989	50	1039	1479
989	228	1217	1657
1100	650	1750	2190
1100	850	1950	2390

PCR products were amplified from plasmid construct PC2 which has no 16S flanking sequences, and contains the intact *npt*II and *gfp* genes. PCR products used as donors span a 434 bp deleted region in the recipients which inactivates both *gfp* anf *nptII* genes and, thus they are able to rescue the two markers in the recipients carrying the inactivated *gfp nptII* constructs (pUCP24-BC2 and BC1). Donors have varying lengths of homology flanking this 434 bp region as shown. Total homology = left homology plus right homology.

DNA PC1 and carrying construct PC2 and 15 μg.mL^{-1} gentamicin for donor DNA carrying construct BC3. Typically, an excess of donor DNA (1 μg in 100 μL) was added to the filter and allowed to dry before 100 μL of recipient culture was applied (10^8 cells). Recipient cultures were grown overnight in LB broth with shaking (120 rpm) at 30 °C; cell numbers were determined by OD_{670} and diluted in Ringer's solution (Fisher Scientific, Loughborough, UK) to 10^9 per mL. Bacterial lysate (prepared as described earlier) or genomic DNA of donor *A. baylyi* strains, PCR product and circular or linearised plasmid DNA of donor constructs were used as donor DNA. As a control, 100 μL of recipient culture was added to a nitrocellulose filter without addition of donor DNA, and the sterility of the donor DNA lysate was confirmed by plating. The plates were incubated at 30 °C for 24 h. Each filter was aseptically removed, placed in 3 mL of sterile quarter strength Ringers Solution (Fisher), and vortex mixed for 1 min. Serial, 10-fold dilutions to 10^{-7} were plated as triplicate 20 μL drops. For lower transformation frequencies 200 μL to 3 mL (concentrated to 100 μL) of mixture was spread on a selective plate. This concentration procedure is effective and yields appropriately increased proportions of transformants (Rochelle et al., 1988; Williams et al., 1996). Plates were incubated at 30 °C for 48 h before colonies were counted and fluorescence was monitored. For low frequency transformations (<10 colonies per plate) all putative transformants were confirmed by colony PCR using appropriate primers from Table 2, followed by gel electrophoresis to confirm product size. Where more than ten putative transformants were obtained in the experiment, at least five colonies were verified in the same way.

To establish that the transformation protocol was working effectively, chromosomal integration transformation was carried out using a lysate of a rifampicin resistant *A. baylyi* BD413 as a positive control. Transformation occurred at a frequency of $9.3 \pm 1.2 \times 10^{-3}$ (n = 3). Transformation frequencies using uncut chromosomal DNA from *A. baylyi* carrying constructs BC2 (Km-r) and BC3 (Gm-r) were not significantly different, confirming that there was no difference in transformation frequency due to the antibiotic selection used. We also confirmed that DNA concentration was saturating in all experiments by regularly calibrating transformation frequency against DNA concentration.

Statistics and analysis

Transfer frequencies, all expressed as transformants per recipient, obtained using different constructs were compared using one-way and two-way analysis of variance. Where necessary, $\log_{10}$ x + 1 transformations were carried out on the data to achieve normality of residuals and homogeneity of variance. Means were compared with the Tukey-Kramer minimum significant difference (MSD) and sum of squares simultaneous test procedure (Fry, 1993). Significant differences are cited at $P \leq 0.05$ unless otherwise stated. Multiple regression was used to predict transformation frequency using the following predictor variables (n = 37): total fragment size, insert size, left and right homologous flanking region sizes, total homology (calculated as the sum of both flank sizes) and ratios

of total homology and insert size. The $\log_{10}$ of transformation frequency was used as the dependent variable to ensure evenness and because the errors with this transformation were not related to the magnitude of the variable. Various transformations of these variables were used to explore improving the regression equation and evenness of the data. The best regression equation was selected by comparing results from the stepwise, forward, backward elimination, and best subsets regression procedures (Fry, 1993). The best equation was selected on the basis of the R^2 and Mallow's C_p statistics, coupled with the degree of variability in the predicted values and the equation's ability to predict data not used to construct it. Statistical analysis was carried out using Minitab version 14.2 (Minitab Inc, State College, Pennsylvania, USA).

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