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An Efficient Method of Selectable Marker Gene Excision by Xer Recombination for Gene Replacement in Bacterial Chromosomes

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A simple, effective method of unlabeled, stable gene insertion into bacterial chromosomes has been developed. This utilizes an insertion cassette consisting of an antibiotic resistance gene flanked by *dif* sites and regions homologous to the chromosomal target locus. *dif* is the recognition sequence for the native Xer site-specific recombinases responsible for chromosome and plasmid dimer resolution: XerC/XerD in *Escherichia coli* and RipX/CodV in *Bacillus subtilis*. Following integration of the insertion cassette into the chromosomal target locus by homologous recombination, these recombinases act to resolve the two directly repeated *dif* sites to a single site, thus excising the antibiotic resistance gene. Previous approaches have required the inclusion of exogenous site-specific recombinases or transposases in *trans*; our strategy demonstrates that this is unnecessary, since an effective recombination system is already present in bacteria. The high recombination frequency makes the inclusion of a counter-selectable marker gene unnecessary.

Antibiotic resistance or other selectable marker genes are routinely used to select for the chromosomal insertion of heterologous genes or the deletion of native genes by homologous recombination to create new strains of bacteria. The presence of antibiotic resistance genes in the host chromosome reduces the variety of plasmids that can be propagated in a cell, since these often rely on the same genes for their selection and maintenance. Genetically modified bacteria containing chromosomal antibiotic resistance genes are undesirable for biotherapeutic production, because the chromosomal DNA will represent a low-level contaminant of the final product and carry the risk of antibiotic resistance gene transfer to pathogenic bacteria in the patient or the environment. The insertion of a constitutively expressed marker gene can also alter the expression of adjacent chromosomal genes. Therefore, a rapid method of inserting genes into or deleting genes from bacterial chromosomes without leaving antibiotic resistance or other selectable marker genes behind is a significant advantage.

One strategy for unlabeled (i.e., without a selectable marker gene) chromosomal gene integration relies on inserting a plasmid via a single homologous recombination event, followed by the removal of the plasmid by a second recombination event (resolution) to hopefully produce the desired genotype (15, 17). A major disadvantage of this approach is that if the insertion or deletion reduces the fitness of the cell, the resolution event will predominantly regenerate the wild-type rather than the mutant genotype and therefore can be inefficient.

An alternative method is to insert an antibiotic resistance gene flanked by regions of chromosomal homology, where recognition sites for a site-specific recombinase (SSR) immediately flank the antibiotic resistance gene. Chromosomal integration strategies include traditional RecA-mediated homologous recombination and recombineering using PCR products and phage-encoded recombination functions, including ET cloning that utilizes RecE/RecT from bacteriophage *Rac* or bacteriophage λ Red recombination (7). Examples of SSRs/target sites used for antibiotic gene excision include Cre/*loxP* from bacteriophage P1 (14), Xis/*attP* from bacteriophage λ (30), and FLP/*FRT* (8) and R/*RS* (26) from the yeasts *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii*, respectively. The recombination functions of transposons such as Tn*4430* from *Bacillus thuringiensis* can also be employed (21). All these strategies require an exogenous recombinase or transposase to be expressed for use in a range of bacteria.

The method described here enlists the Xer recombinases that are naturally present in bacteria to excise antibiotic resistance genes following chromosomal integration, thereby eliminating the requirement for an exogenous SSR system. These are XerC and XerD in *Escherichia coli* (16), with homologues in many other species, such as RipX and CodV in *Bacillus subtilis* (22). The antibiotic resistance gene is flanked by the 28-bp *dif* sites, which enable Xer recombinases to resolve the chromosome and plasmid dimers generated by RecA in many prokaryotes that possess circular replicons. Therefore, intramolecular Xer recombination will excise a *dif*-flanked antibiotic resistance or other selectable marker gene from a chromosomally inserted cassette (Fig. 1). Here we demonstrate the use of this technology for antibiotic resistance gene removal by the deletion of chromosomal genes and the insertion of an exogenous gene. The frequency of gene excision by Xer recombination was also demonstrated in *E. coli* and *B. subiltis*.

MATERIALS AND METHODS

DNA manipulation. *Pfu* DNA polymerase (Stratagene) was used to generate PCR products for cloning and gene insertion applications, and ReddyMix was used (Abgene) for screening of colonies by PCR, with standard PCR protocols employed for all reactions. Restriction enzymes (New England Biolabs), T4 DNA ligase, and the Wizard Genomic DNA Purification kit (Promega) were used according to the manufacturers' instructions.

Bacterial strains and media. *E. coli* DH1 (11) and *B. subtilis* 168 (13) were the target strains for the gene integration experiments. *E. coli* and *B. subtilis* were cultured in Luria-Bertani (LB) medium and Antibiotic Medium 3 (AM3; Difco), respectively, in liquid broth and 1.5% agar plates containing the following con-

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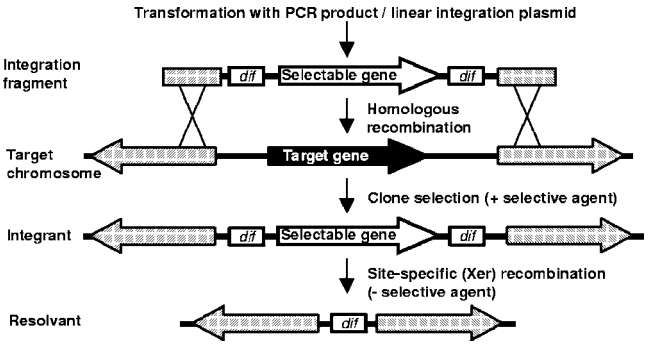

FIG. 1. Deletion of a target chromosomal gene and subsequent removal of the selectable marker (e.g., antibiotic resistance) gene by Xer recombination at flanking *dif* sites (Xer-cise). Shaded regions represent homology between the integration cassette and genes flanking the target gene.

centrations of antibiotics where required: 100 μg ml^{-1} ampicillin, 12 μg ml^{-1} tetracycline, or 20 μg ml^{-1} chloramphenicol (10 μg ml^{-1} chloramphenicol for *B. subtilis*). All bacterial cultures were incubated at 37°C, with shaking at 200 rpm for liquid cultures. Strains and plasmids used in this study are described in Table 1.

Bacterial transformation. Electrocompetent *E. coli* cells were produced by the method of Seidman et al. (23). Competent *B. subtilis* cells were produced by an optimized two-step method, which involved growth in a transformation medium containing 100 mM potassium phosphate buffer, 2% glucose, 0.2% potassium L-glutamate, 11 mg liter^{-1} ammonium iron(III) citrate, 80 mg liter^{-1} L-tryptophan, 3 mM trisodium citrate, 3 mM $MgSO_4$, and 0.1% casein hydrolysate. The culture was inoculated at an A_{500} of 0.1, grown to an A_{500} of 1.3, then diluted with an equal volume of transformation medium without casein hydrolysate, and incubated for 1 h. Cells were centrifuged and resuspended in the supernatant at 1/8 of the culture volume, and resuspension buffer containing 100 mM potassium phosphate buffer, 0.5% glucose, and 20 mM $MgSO_4$ was added at 1/6 of the resuspended cell volume. DNA was added to 400-μl aliquots and incubated for 1 h; then an expression mixture containing 2.5% yeast extract, 2.5% casein hydrolysate, and 0.4 g liter^{-1} L-tryptophan was added, and the mixture was incubated for a further hour prior to plating on selective agar.

***E. coli* chromosomal gene integration.** The chloramphenicol resistance gene *cat* was amplified from plasmid pKO3 (17) using primers 5DifCAT and 3DifCAT. (All primers are described in Table 2.) These primers incorporated a 3′ region of homology flanking the *cat* gene in pKO3 with a 5′ tail that included a 28-bp *E. coli dif* site ($dif_{E.\ coli}$; GGTGCGCATAATGTATATTATGTTAAAT) and Bsu36I and NsiI restriction sites. This PCR product was cloned into pCR2.1-TOPO (Invitrogen) to create a precursor gene deletion plasmid, pTOPO-DifCAT.

To delete *msbB* from the *E. coli* chromosome, the $dif_{E.\ coli}$-*cat*-$dif_{E.\ coli}$ cassette from pTOPO-DifCAT was amplified by PCR using 70-nucleotide (nt) primers msb.int F and msb.int R (50 nt of the 5′ ends was homologous to the chromosomal regions flanking *msbB*, and 20 nt of the 3′ ends was homologous to pTOPO-DifCAT). DH1 was first transformed with the tetracycline-selectable plasmid pTP223, which provides the λ Red gene functions for protection and integration of linear DNA (18), Electrocompetent DH1(pTP223) was transformed with the $dif_{E.\ coli}$-*cat*-$dif_{E.\ coli}$ PCR product, and integrants were selected on LB agar containing chloramphenicol. Subculturing in LB broth in the absence of antibiotics resulted in the loss of pTP223 and the generation of chloramphenicol-sensitive recombinant clones, which were identified by replica streaking onto agar plates with and without the selective antibiotic and screened by PCR using primers SML and SMR.

The *rbpA* gene from pQR163 (27) was cloned as an SphI-SmaI fragment adjacent to Bsu36I $dif_{E.\ coli}$-*cat*-$dif_{E.\ coli}$ from pTOPO-DifCAT into prbpA-DifCAT. This was used as a PCR template with the 50-bp 5′ ends of the 70-nt primers Int F and Int R homologous to the *ubiB-fadA* target locus. The PCR integration fragment was transformed into electrocompetent DH1(pTP223) to create the integrant strain. Plasmid-free recombinant clones were selected as described above and screened by PCR using primers Ubi F and Ubi R.

***B. subtilis* chromosomal gene integration.** The chloramphenicol resistance gene *cat* was amplified from plasmid pypmP::CAT using primers 5bac.DifCAT and 3bac.DifCAT. These primers incorporated a 3′ region of homology flanking the *cat* gene and a 5′ tail that included a 28-bp *B. subtilis dif* site ($dif_{B.\ subtilis}$; ACTTCCTAGAATATATATTATGTAAACT) and the AgeI and BstEII restriction sites. This PCR product was cloned into pCR2.1-TOPO to create a precursor gene deletion plasmid, pTOPO-bac.DifCAT.

For deletion of the majority of the cistrons of extracellular protease genes *mpr* and *nprE* from *B. subtilis* 168, these genes were amplified by PCR from chromosomal DNA including ~0.4 kb of homology flanking the regions to be deleted (using primer sets SEQ5MPR-SEQ3MPR and YKTD-SEQ3NPRE) and cloned into pCR2.1-TOPO to create pTOPO-mpr and pTOPO-nprE. *mpr* was removed by AgeI-SspI digestion and replaced with an AgeI-BfrBI $dif_{B.\ subtilis}$-*cat*-$dif_{B.\ subtilis}$ fragment from pTOPO-bac.DifCAT to create deletion plasmid pmpr-DifCAT. *nprE* was removed by BstEIII-SspI digestion and replaced with a BstEII-BfrBI $dif_{B.\ subtilis}$-*cat*-$dif_{B.\ subtilis}$ fragment from pTOPO-bac.DifCAT to create deletion plasmid pnprE-DifCAT. The deletion plasmids were linearized by restriction digestion in the cloning vector and transformed into competent *B. subtilis* 168, and integrants were selected on AM3 agar containing 10 μg ml^{-1} chloramphenicol. Subculturing in AM3 broth in the absence of antibiotics produced chloramphenicol-sensitive recombinant clones, identified by replica plating onto agar with and without the selective antibiotic. Putative recombinants were screened by PCR using primer set C5MPR-C3MPR or C5NPRE-C3NPRE, which flanked the deletion sites.

Estimation of excision frequency at *dif* sites. Triplicate cultures of integrant strains containing chloramphenicol were used to inoculate 50-ml shake flasks without chloramphenicol to a starting A_{600} of 0.005. After each 24-h period, the number of generations reached was calculated and the cultures inoculated into fresh medium. Following further rounds of daily subculturing (total growth times, 48 and 96 h for *E. coli* and 24 and 48 h for the *B. subtilis* strains), the total number of generations throughout the experiment was calculated, and cultures were serially diluted and plated onto nutrient agar to give single colonies. One hundred colonies derived from each culture were plated onto fresh nutrient agar with or without chloramphenicol. Clones that had become chloramphenicol sensitive were screened by PCR using diagnostic primers listed in Table 2. The resulting data were used to calculate the excision frequencies.

RESULTS

Gene integration and selectable marker excision. The *msbB* gene product attaches the immunogenic myristoyl group to lipid A in the cell membrane (25), so *msbB* was deleted from the *E. coli* DH1 chromosome to generate a new strain with reduced endotoxin activity suitable for plasmid DNA production (Fig. 2A). A plasmid with the *cat* gene flanked by *dif* sites, pTOPO-DifCAT, was used as a template for assembling a deletion cassette by PCR using primers with 5′ homology to the target locus flanking the region to be deleted. PCR screen-

TABLE 1. Bacterial strains and plasmids used directly in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
DH1	F$^-$ *supE44 hsdR17 recA1 gyrA96 relA1 endA1 thi-1* λ$^-$	11
DH1M	DH1 *msbB*	This work
DH1R	DH1::*rbpA*	This work
B. subtilis		
168	*trpC2*	13
168-mpr	*trpC2 mpr*	This work
168-nprE	*trpC2 nprE*	This work
Plasmids		
pCR2.1-TOPO	Vector for cloning PCR products	Invitrogen
pKO3	Source of *cat* in pTOPO-DifCAT	17
pTOPO-DifCAT	Precursor *E. coli* Xer-cise plasmid	This work
pQR163	Source of *rbpA*	27
prbpA-DifCAT	Precursor *rbpA* integration Xer-cise plasmid	This work
pTP223	Lambda Red helper plasmid	18
pypmP::CAT	Source of *cat* in pTOPO-bac.DifCAT	G. Homuth
pTOPO-bac.DifCAT	Precursor *B. subtilis* Xer-cise plasmid	This work
pmpr-DifCAT	*mpr* deletion Xer-cise plasmid	This work
pnprE-DifCAT	*nprE* deletion Xer-cise plasmid	This work

TABLE 2. PCR primers used in this study

Name	Size (nt)	Sequence (5′→3′)[a]	Function of PCR product
5DifCAT	81	CCTTAGGATGCATGGTGCGCATAATGTATATTATGTTAAATCCCTTAT **GCGACTCCTGCATCCCTTTCGTCTTCGAATAAA**	$dif_{E.\ coli}$-*cat*-$dif_{E.\ coli}$ for pTOPO-DifCAT
3DifCAT	81	CCTTAGGATGCATATTTAACATAATATACATTATGCGCACCATCCGCTT **ATTATCACTTATTCAGGCGTAGCACCAGGCGT**	
5bac.DifCAT	84	AATACCGGTGGTGACCACTTCCTAGAATATATATTATGTAAACTTATT **CTTCAACTAAAGCACCCATTAGTTCAACAAACGAAA**	$dif_{B.\ subtilis}$-*cat*-$dif_{B.\ subtilis}$ for pTOPO.bac-DifCAT
3bac.DifCAT	77	TTGAATATTAGTTTACATAATATATATTCTAGGAAGTGATATCTTCAAC **TAACGGGGCAGGTTAGTGACATTAGAAA**	
msb.int F	70	TGCGGCGAAAACGCCACATCCGGCCTACAGTTCAATGATAGTTCA ACAGA**AGTGTGCTGGAATTCGCCCT**	*msbB* deletion
msb.int R	70	TTGGTGCGGGGCAAGTTGCGCCGCTACACTATCACCAGATTGATT TTTGC**ATCTGCAGAATTCGCCCTTA**	
Int F	70	AAACCCGCCCCTGACAGGCGGGAAGAACGGCAACTAAACTGTTAT TCAGT**TTGCGCCGACATCATAACGG**	*rbpA* insertion
Int R	70	GCCGGATGCGGCGTGAACGCCTTATCCGGTCTACCGATCCGGCAC CAATG**GCTACGGTTTGATTAGGGAA**	
SEQ5MPR	20	TGTTGAAGGATTGGAAAACG	*mpr* cloning locus
SEQ3MPR	20	ACTAATGGAATGGCATGATC	
YKTD	21	AATTAGAGACGTTAAGCTGGA	*nprE* cloning locus
SEQ3NPRE	20	ATACATAATGACTGAATAAC	
SML	20	TGACCTGGTGATTGTCACCC	*msbB* screening locus
SMR	20	TAAACCAGCAGGCCGTAAAC	
UbiB F	20	GATCGCCTGTTTGGCGATGC	*ubiB-FadR* screening locus
UbiB R	20	GAATCTGATGGAACGCAAAG	
C5MPR	20	TTTCGAATCAGAAATCACAC	*mpr* screening locus
C3MPR	20	CTACTCTTTCAGGCGCGCGG	
C5NPRE	20	TGATCAACCTCGAAAACCTG	*nprE* screening locus
C3NPRE	20	GTATATGGCATTACTGCACC	

[a] For primers 5DifCAT and 3DifCAT, the $dif_{E.\ coli}$ sites are double underlined and the *cat* homology regions are boldfaced. For primers 5bac.DifCAT and 3bac.DifCAT, the $dif_{B.\ subtilis}$ sites are double underlined and the *cat* homology regions are boldfaced. For primers msb.int F and msb.int R, the homology regions flanking *msbB* are underlined and the pTOPO-DifCAT homology regions are boldfaced. For primers Int F and Int R, the homology regions flanking *ubiB*/*fadR* are underlined and the prbpA-DifCAT homology regions are boldfaced.

ing using primers SML and SMR to amplify a part of the *msbB* locus (Fig. 2B) gave a product of 1,428 bp for the native *msbB* locus of DH1 (lane 2). The integrant locus was 1,460 bp, but this PCR also amplified a product of 462 bp, indicating an *msbB* deletion, as a proportion of the population underwent Xer recombination even in the presence of chloramphenicol (lane 3). This could be occurring in the later stages of growth as the antibiotic is degraded and the selection pressure reduced. The PCR of the recombinant, *msbB*-deleted strain DH1M, shows only the 462-bp deletion product (lane 4).

The bovine pancreatic RNase gene *rbpA* was inserted into a chromosomal space between two native genes (*ubiB* and *fadA*) in *E. coli* DH1 that had been chosen arbitrarily (Fig. 2C). This insertion created DH1R, a *recA* equivalent of a previous strain, JMRNaseA (4), that exports RNase A to the periplasm, where it folds to the active conformation and is then released to degrade RNA upon cell lysis. PCR using primers UbiB F and UbiB R, flanking the integration region in DH1, gave a product of 510 bp for the native *ubiB-fadA* locus (Fig. 2D, lane 2). The integrant locus gave 1,936 bp, but this PCR also amplified a product of 938 bp, indicating deletion of *cat*, as a proportion of the population underwent Xer recombination even in the presence of chloramphenicol (lane 3). The integrated *rbpA* gene without *cat* was detected as a 938-bp PCR product in DH1R (lane 4).

B. subtilis secretes proteases into the growth medium, and these can have the adverse effect of degrading secreted recombinant proteins. Two of these extracellular protease genes, *mpr* (24) and *nprE* (28), were chosen to test *dif*-flanked selectable marker gene deletion. The PCR results were equivalent to those seen for *E. coli* and so are not shown; as with *E. coli*, the integrant strains cultured in the presence of chloramphenicol also displayed the recombinant PCR products. Diagrams of the extracellular protease gene loci in wild-type *B. subtilis* 168 and the deleted loci in 168-mpr and 168-nprE are shown in Fig. 3.

Excision frequency at *dif* sites. The percentages of integrant cells that had undergone Xer recombination to excise the chromosomal *cat* gene at selected time points are given in Table 3. Results were determined by subculturing for a time sufficient to identify a recombinant clone if 100 colonies were replica plated in the presence or absence of the selective antibiotic.

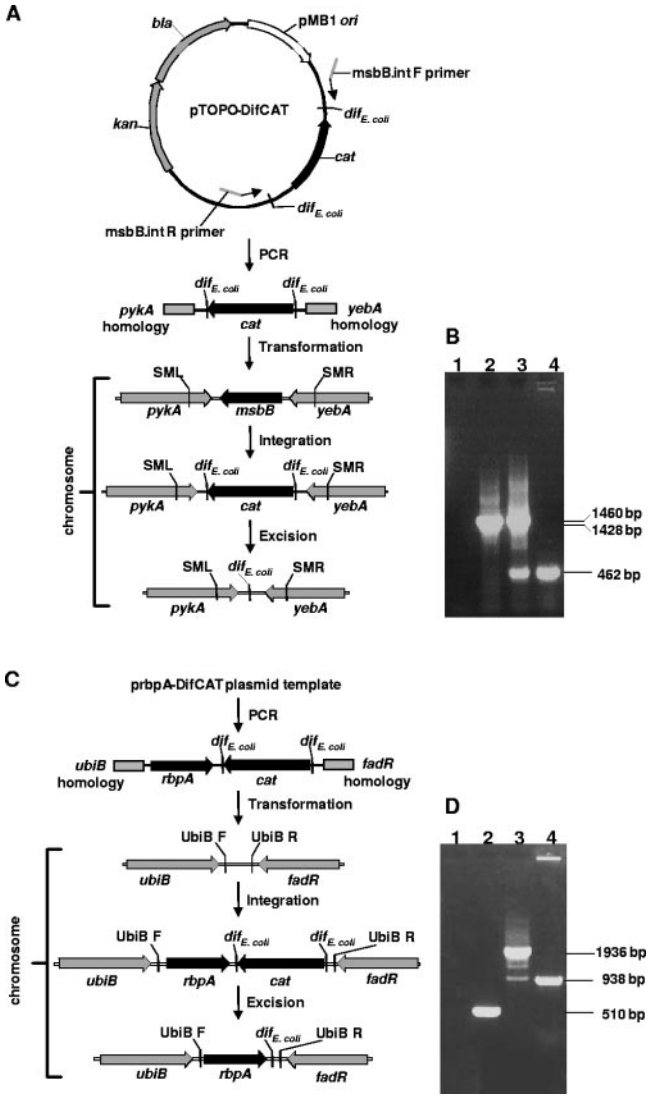

FIG. 2. Integration of PCR products and antibiotic resistance gene excision in *E. coli*. (A) Deletion of *msbB* using a PCR product with homology to the regions flanking chromosomal *msbB* to generate DH1M. (B) Agarose gel of PCR products generated using primers SML and SMR. Lane 1, negative control (no template DNA); lane 2, wild-type *msbB* locus; lane 3, integrant; lane 4, deletion mutant. (C) Chromosomal insertion of *rbpA* using a PCR product with homology to the *ubiB-fadR* intergenic region to generate DH1R. (D) Agarose gel of PCR products generated using primers UbiB F and UbiB R. Lane 1, negative control (no template DNA); lane 2, wild-type *ubiB-fadR* locus; lane 3, integrant; lane 4, recombinant with inserted *rbpA*.

With *E. coli*, this time was 48 h after inoculation, but with the significantly higher frequency of Xer recombination in *B. subtilis*, a high proportion of cells had resolved after 24 h. All chloramphenicol-sensitive colonies were screened by PCR to ensure that this phenotype was due to *cat* excision and not to mutation.

DISCUSSION

We have demonstrated a simple, rapid technique for selectable marker gene removal following gene integration in bacteria that should be applicable to all prokaryotes with the ubiquitous Xer dimer resolution system (20). A cassette consisting of an antibiotic resistance gene flanked by *dif* sites and regions of homology to the chromosomal target is amplified by PCR or cloned into a plasmid and then integrated into the chromosome. Cells that have undergone intramolecular Xer recombination at *dif* sites during further culture (and therefore have lost the resistance gene) are identified by antibiotic sensitivity and verified by PCR. We propose the term "Xer-cise" to describe this technique.

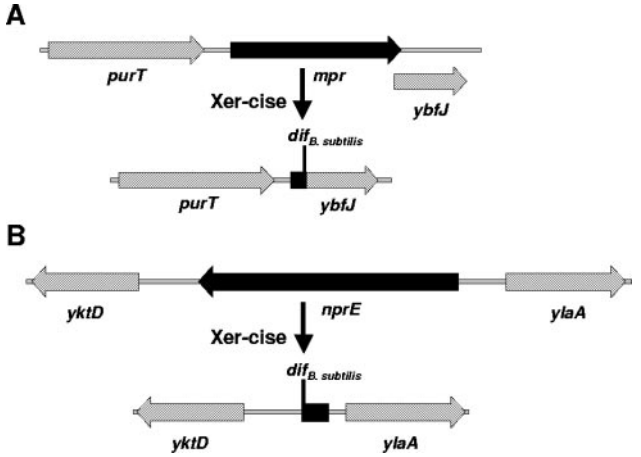

FIG. 3. Wild-type and deleted loci of extracellular protease genes *mpr* (A) and *nprE* (B) in *B. subtilis*.

With unlabeled gene replacement technologies that rely on homologous rather than site-specific recombination for removal of the integrated cassette, a gene that allows positive selection is required (usually adjacent to the antibiotic resistance gene), since the homologous recombination frequency is too low to make screening by replica plating a viable method of identifying recombinant clones. An example is *sacB*, which expresses the *B. subtilis* enzyme levansucrase. This enzyme converts sucrose into levans, which are lethal to *E. coli* (17). If an integrant that has been cultured in the absence of the selective antibiotic is plated on a medium containing sucrose, any cell that has not lost both genes (by a second homologous recombination event) will be killed. A counter-selectable marker gene used in *B. subtilis* to identify recombinants is the *upp* gene encoding uracil phosphoribosyltransferase, which is toxic on media containing 5-fluorouracil, but *upp* must be deleted from the chromosome before this system can be used (10). The frequency of Xer recombination is high enough to detect recombinant clones by replica plating a small number of colonies onto agar plates with and without the selective antibiotic, making the inclusion of a counter-selectable gene unnecessary. This is advantageous, since certain counter-selectable genes such as *sacB* can be mildly toxic even when counter-selection is not present, leading to an accumulation of cells carrying mutations in *sacB* and therefore generating false-positive results during clone selection.

Because recombination frequency is independent of the presence of a selective antibiotic, integrant cells will undergo Xer recombination even if the antibiotic is present (Fig. 2, lanes 3), but the resulting recombinants will be killed. We do

TABLE 3. Gene excision frequencies by Xer site-specific recombination at *dif* sites

Parameter	Value for the following locus at the indicated time (h):							
	E. coli msbB		*E. coli ubiB-fadA*		*B. subtilis mpr*		*B. subtilis nprE*	
	48	96	48	96	24	48	24	48
No. of generations[a,b]	19.7 ± 0.1	39.2 ± 0.1	18.7 ± 0.2	37.9 ± 0.2	8.4 ± 0.1	15.4 ± 1.4	8.4 ± 0.1	15.8 ± 1.3
% Recombinants[b,c]	6.3 ± 1.5	7.7 ± 2.3	1.0	4.6 ± 3.2	41.3 ± 4.7	68.7 ± 8.1	22.0 ± 2.6	48.3 ± 4.5
Excision frequency[d]	3.2×10^{-3}	2.0×10^{-3}	5.3×10^{-4}	1.2×10^{-3}	0.05	0.04	0.03	0.03

[a] Calculated as $(\ln N_t - \ln N_0)/\ln 2$ where N_t is the final optical density and N_0 is the optical density of the inoculum (absorbance at 600 nm) at each 24-h subculture point.
[b] Results are means and standard deviations for simultaneous experiments on three integrant clones.
[c] The percentage of cells within the population that have undergone a recombination event to excise the *cat* gene.
[d] The proportion of recombinants per generation (percentage of recombinants/100 × number of generations).

not report analyses of the modified phenotypes of the recombinant strains here, because we are focusing on gene integration and excision, but in addition to the diagnostic PCRs, the chromosomal insertion sites of recombinant clones were sequenced, confirming that a single *dif* site had been generated.

The natural *dif* site is in the chromosome terminus region and is essential for correct chromosome segregation at cell division: deletion of $dif_{E.\ coli}$ results in a subpopulation of *E. coli* cells with a filamentous morphology (12). However, if natural $dif_{E.\ coli}$ is deleted, another $dif_{E.\ coli}$ site will not enable dimer resolution if placed outside of the ~30-kb *dif* activity zone (DAZ) in the terminus region (6, 16). Therefore, while our method involves insertion of one or more extra *dif* sites at different chromosomal loci, this should not have an adverse effect on chromosome segregation, although the possibility that introduction of multiple *dif* sites in close proximity might result in deletion of intervening chromosomal DNA should be considered.

The efficiency of Xer recombination at tandemly repeated *dif* sites within the *E. coli* DAZ has been determined by Barre et al. (1) using a $dif_{E.\ coli}$-flanked kanamycin resistance gene, and more than 90% of cells of a RecA$^-$ strain became kanamycin sensitive following overnight culture (99% for a RecA$^+$ strain). This is a significantly higher percentage than we have observed in our RecA$^-$ examples outside of the DAZ (1.0 and 6.3% after 48 h). However, the frequencies that we report still allow the required recombinant mutants to be easily identified by screening a small number of colonies for antibiotic sensitivity and may be higher in a RecA$^+$ strain. The recombination frequency in *B. subtilis* is significantly higher than that in *E. coli*, suggesting that intramolecular recombination by RipX/CodV is more efficient than that by XerC/XerD, although the examples presented here compare RecA$^-$ *E. coli* and RecA$^+$ *B. subtilis* strains. The deleted *B. subtilis* protease genes were not in a region equivalent to the DAZ, and the *dif*-flanked antibiotic resistance genes were of equivalent lengths in *E. coli* and *B. subtilis* integration plasmids, so these factors cannot be responsible for the differences in Xer recombination frequency between the two species. The Xer recombination frequency per generation (Table 3, Excision frequency) remained constant in all examples except for *E. coli ubiB-fadA*, indicating that repeated subculturing should increase the probability of identifying a recombinant.

It should be noted that although *dif*-containing plasmids can be integrated into the chromosomal *dif* site (19), this is a different approach in which the entire plasmid is integrated by Xer recombination and excised by the same mechanism, so the plasmid is unstable in the absence of antibiotic selection pressure.

We have used chromosomal *dif* sites as the substrates for intramolecular Xer recombination, but the equivalent plasmid dimer resolution sites, such as the *cer* or *psi* sites or hybrids thereof that function in *E. coli*, may also be suitable. These require accessory sequences for recombination on plasmids but not for chromosomal recombination (5). Intramolecular recombination will occur between two *dif* sites on plasmids (2, 19), but culturing in a medium containing chloramphenicol prevented this from being a problem with our precursor plasmids. However, excision of plasmid-borne genes is an alternative application of Xer-cise and may be used to regulate gene expression or excise antibiotic resistance genes when used with antibiotic-free plasmid maintenance systems.

We have used PCR primers containing 50-bp regions of homology to the chromosomal target for λ Red integration, since short homologous sequences are known to work well in *E. coli* (8). For *B. subtilis* we used linearized plasmids with inserts containing approximately 400 bp of target locus homology, but we have not yet attempted to use homology regions of a size that can be synthesized as primers. Splicing PCR can also be used to assemble integration cassettes with long regions of homology as an alternative to plasmid construction. The λ Red functions encoded on pTP223 were used to enable insertion of linear fragments in *E. coli*, since Gam inhibits linear DNA degradation by the native RecBCD exonuclease, while Beta and Exo enable chromosomal integration (18), essential in RecA$^-$ strains. For alternative integration methods, ET recombination utilizing RecE and RecT could be used in *sbcA E. coli* strains (29), or it may be possible to use electroporation for chromosomal integration of linear DNA without the need for λ Gam (9). With *B. subtilis*, no additional functions are required in *trans* to mediate homologous recombination, since it does not significantly degrade linear DNA following transformation.

We anticipate that the Xer-cise technique of selectable marker gene excision at *dif* sites will simplify and accelerate the production of unlabeled mutants in *E. coli* and *B. subtilis* and will be applicable to the ever-increasing number of bacteria for which the native *dif* site has been elucidated (3). It should also be possible to use the Xer recombinase genes in *trans* to enable selectable marker gene removal in eukaryotes.

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