An improved system for gene replacement and xylE fusion analysis in Pseudomonas aeruginosa

(Blue-white screening; sacB; conjugation; xylE cassette; gentamycin resistance)

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Received by A.M. Chakrabarty: 14 October 1994; Revised/Accepted: 1 December/12 December 1994; Received at publishers: 13 January 1995

SUMMARY

A novel pUC19-based gene replacement vector has been developed. This vector incorporates (i) the counterselectable sacB marker, (ii) a lacZ~ allele for blue-white screening, (iii) an oriT for conjugation-mediated plasmid transfer and (iv) unique cloning sites for SmaI and the rare-cutting meganuclease I-SceI. These rare restriction sites are also present on the helper plasmid pUCim9Sce. The replacement vector is engineered to contain few restriction sites to gain greater access to restriction sites within cloned DNA fragments, thus facilitating their genetic manipulation. The usefulness of the system was demonstrated by chromosomal integration of a newly constructed xylE::Gm fusion cassette into the glpD gene of Pseudomonas aeruginosa.

INTRODUCTION

Our laboratory has recently described the first gene replacement system for Pseudomonas aeruginosa (Pa) which allowed positive selections for all steps involved in the gene replacement process (Schweizer, 1992). The most significant improvement was the inclusion of the B. subtilis sacB gene which allowed for positive selection of the segregation of true mutants from the more frequently occurring merodiploids. This selection process greatly facilitates the genetic exchange not only of selectable markers but also screenable markers, e.g., lacZ- or xylE-based gene fusions (Schweizer, 1993a). The replacement system is useful, but it suffers some drawbacks which lie mostly in the multiple cloning steps involving specialized vectors that underlie the procedure as previously detailed (Schweizer, 1992). For even greater flexibility of the sacB-based gene replacement system, a vector should be avail-

Abbreviations: A, absorbance (1 cm); aa, amino acid(s); Ap, ampicillin; B., Bacillus; bia, gene encoding β-lactamase; bp, base pair(s); βGal, β-galactosidase; CAA, casamino acids; Cb, carbencillin; Cm, chloramphenicol; C230, catechol 2,3-dioxygenase; Δ, deletion; E., Escherichia; Gm, gentamicin; G3P, sn-glycerol-3-phosphate; G3PDH, G3P dehydrogenase; glpD, G3PDH-encoding gene; IPTG, isopropyl-β-D-thio-galactopyranoside; kb, kilobase(s) or 1000 bp; lacI, gene encoding lac repressor LacI; lacI promoter; lacIq, lacI promoter with up-mutation q; lacZa, gene encoding α peptide of βGal; lacZΔM15, gene encoding M15 peptide of βGal; lacZpo, lac promoter-operator; LB, Luria-Bertani (medium); MCS, multiple cloning site(s); nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ONPG, o-nitrophenol-β-D-galactopyranoside; oriT, origin(s) of transfer; P., Pseudomonas; Pa, P. aeruginosa; PIA, Pseudomonas isolation agar; Pol, DNA polymerase; Polk, Klenow (large) fragment of E. coli Polk; r, ribosomal; s, resistance/resistant; sensitive/sensitivity; sacB, levansucrase-encoding gene; Suc, sucrose; TAE, Tris-acetate buffer (40 mM Tris-acetate pH 8.5/1 mM EDTA); tet, tetracycline-efflux protein-encoding gene; tetp, tet promoter; Tm, melting temperature; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; xylE, gene encoding C230; [], denotes plasmid-carrier state; ::, novel junction (fusion or insertion); ' (prime), denotes a truncated gene at the indicated side.

SSDI 0378-1119(95)00055-0
able containing few restriction sites within the vector to give greater access to restriction sites within the cloned fragment, thus allowing the construction of insertions, deletions and frameshifts. In addition, the oriT and sacB should be present on the same vector, thus eliminating an additional cloning step and expediting the procedure. As a first step towards this goal, Quandt and Hynes (1993) recently described a vector, pJQ200uc1, which fulfills some of the criteria outlined above. However, this vector still possesses some properties which can make it difficult to use in routine allele replacement: (i) although the vector contains a single SmaI site for cloning of DNA fragments, restriction sites for EcoRI, HindIII and KpnI located within sacB, plus other sites located throughout the vector sequences, still compromise accessibility to these sites within cloned DNA; (ii) the vector utilizes GmR as the selectable marker. Since GmR is one of the few markers useful for insertion mutagenesis in Pa (Schweizer, 1993b), this makes the vector in its present form less attractive for use in this bacterium and other bacteria with a similar limited range of useful antibiotics.

The goals of the studies presented in this paper were twofold: First, to construct an improved gene replacement system addressing the aforementioned issues. Second, to extend the range of available genetic markers for chromosomal analysis in conjunction with the improved gene replacement system. This was achieved by construction of a selectable xylE-GmR cassette that has the advantage of allowing mutational analysis by insertion inactivation while simultaneously constructing transcriptional xylE gene fusions.

RESULTS AND DISCUSSION

(a) Construction of pUC19Sce

The goal was to insert a I-SceI-SmaI-I-SceI linker into the pUC19 (Yanisch-Perron et al., 1985) derivative pUC1918 (Schweizer, 1993a) in a manner that would still allow blue-white screening for inserts without positive selection. This was achieved by designing a pair of oligos that, after cloning between the HindIII sites of pUC1918 in the correct orientation, allowed plasmid-directed synthesis of an in-frame, active βGal α peptide (Fig. 1B). The presence of the I-SceI and SmaI sites in the pUCSce polylinker was verified by restriction mapping and by nt sequencing. The plasmid allows (i) monitoring of successful cloning of fragments into the SmaI site by blue-white screening in the appropriate host strain, e.g., DH5αF’ (Liss, 1987); and (ii) rescue of the cloned DNA fragment after the desired manipulation(s) by I-SceI digestion which recognizes a rare 18-bp nt sequence (Monteilhet et al., 1990; Fig. 1B) which to this date has not been found in bacterial DNA (GenBank release 86.0, December 15, 1994).

(b) Construction of replacement vector pEX100T

The assembly of pEX100T was achieved in several steps (Fig. 2). First, the EcoRI, HindIII and KpnI sites located within the sacB coding sequence were eliminated by site-directed mutagenesis (Lewis and Thompson, 1990) utilizing primers introducing single nt substitutions without affecting codon usage. Second, an oriT was placed next to the modified sacB gene. Third, the oriT and sacB sequences were cloned into pUCSce. In the final step, the double transcriptional terminators from the E. coli rnrB operon (Brosius et al., 1981) were inserted downstream from lacZpo for increased plasmid stability. The resulting vector pEX100T possesses some unique features which make it extremely user-friendly for routine one-step allele replacement procedures: (i) unlike pJQ200uc1 (Quandt and Hynes, 1993) which in our...
Fig. 2. Construction of pEX100T. Plasmids are drawn to scale. The location of genes and their transcriptional orientation are shown; \( \text{bla}^* \), encodes the frame-shift (Ap\(^+\)) containing \( \text{bla} \) gene from pALTER-1 (Lewis and Thompson, 1990); \( \text{ori} \), pMB1-based origin of replication (Balbás et al., 1986); \( \text{oriT} \), origin of transfer from pEM5 (Sonnen et al., 1991); \( \text{tet} \), tetracycline efflux protein-encoding gene from pALTER-1 (Lewis and Thompson, 1990); \( \text{sacB} \), levansucrase-encoding gene from pMOB2 (Schweizer, 1992); \( \text{T} \), double transcriptional terminators \( T_1 \) and \( T_2 \) and 5S rRNA gene from pTT\( \Delta \)AS (Schweizer, 1991a). The nt sequence of pEX100T can be obtained from GenBank under accession No. U17500. **Methods:** For construction of pALB1, a 2.6-kb \( PstI-XbaI \) fragment from pMOB2 was gel-purified (Wieslander, 1979) and ligated to \( PstI + XbaI \)-digested pALTER-1 DNA. The construction of the gene replacement vector pEX100T was achieved in several steps: (A) pALB2 was obtained by mutagenizing (Lewis and Thompson, 1990) the EcoRI, HindIII and KpnI sites located in \( \text{sacB} \) utilizing the following oligos designed to introduce single nt substitutions (underlined): \( \text{PAEcoRI} \), 5'-CAAGTTCCTGAGTTCGATTCGTCC; \( \text{PAHindIII} \), 5'-GCGCCTAGCTTCCTGCTGAAC; \( \text{PAKpnI} \), 5'-GGCAAATGGTATCTGTTCACTGAC; (B) for construction of pALB3, \( \text{oriT} \) was isolated from pEM5 by \( \text{SalI} + XbaI \) digestion, blunt-ending with T4 DNA Pol and ligation of the resulting 900-bp fragment to pALB2, which had been digested with \( \text{SalI} + XbaI \) and treated with T4 DNA Pol; (C) pEX100 was derived by ligation of a Pollk-treated 1.9-kb \( \text{EcoRV-SalI} \) fragment from pALB3 into the Pollk blunt-ended NdeI site of pUCSce (Fig. 1); (D) pEX100T was obtained as follows: a 500-bp DNA fragment was isolated from pTT\( \Delta \)AS by \( \text{SalI} + \text{HindIII} \) digestion, end repaired with T4 DNA Pol and ligated into the T4 DNA Pol-treated NarI site of pEX100. All \( \text{sacB} \)-containing constructs confer sucrose-sensitivity on E. coli strains when grown on LB medium supplemented with 5% sucrose. For increased plasmid stability, pEX100T was maintained in the LacI overproducing strain HPS1 \{F\(^-\) Δ(lac-proAB) endA1 gyrA96 hsdR17 supE44 relA1 recA1 thi rif\(^R\) zzz::mini-Tn5lacO \[lacZAM15\(^+\) lacI\(^+\) Cm\(^R\)\] \} (Schweizer, 1994).
Plasmid pALX1 was then mutagenized by employing a mutagenic oligo (5'-CAATGTCAGCCGGCAACGGCC) which corresponds to expression which allows for reliable blue/white screening pUC vectors with a stable replicon and consistent

In contrast to pJQ200ucl, the new vector contains no BglII, EeoRI, EcoRV, HindIII, KpnI, NdeI, NotI sites; (iii) in rare cases, the restriction sites still present in the new vector may interfere with the intended cloning procedures. In such cases, pUCSce may serve as an auxiliary plasmid from which the manipulated DNA can be excised by I-SceI digestion without fear of inactivation of cloned DNA segments; (ii) substitution of the GmR -encoding gene with the ApR-encoding gene as the selectable marker still allows for utilization of the previously constructed GmR cassettes (Schweizer, 1993b) as a useful marker in insertion mutagenesis in Pa. If necessary, the ApR marker can easily be replaced by cloning other antibiotic-resistance-encoding gene cassettes into the unique Scal site located within the bla gene.

**Methods:** (A) For construction of pX1918G, a 1440-bp xylE-containing EcoRI fragment was isolated from pX1918 (Schweizer, 1993a) and cloned into the EcoRI site of pALTER-1 (Lewis and Thompson, 1990) (pALX1). Plasmid pALX1 was then mutagenized by employing a mutagenic oligo (5'-CAATGTCAGCCGGCAACGGCC) which corresponds to nt 1680–1710 of pX1918 (GenBank accession No. U03992), with the exception of the underlined C→G change, and a previously described mutagenesis procedure (Lewis and Thompson, 1990). This procedure generated a unique Scal site 108 nt downstream from xylE (pALX2). Next, the pALTER-1 MCS Scal site was eliminated from pALX2 by Sassl XbaI digestion, treatment with T4 DNA Pol and religation (pALX3). Plasmid pALX4 was derived from pALX3 by isolating a 830-bp T4 DNA Pol-treated, GmR-conferring SacI fragment from pUCGM and ligation into the unique Scal site located downstream from xylE. Finally, pX1918G was obtained by excision of a 2273-bp EcoRI fragment fragment from pALX4 and ligating it into EcoRI-cleaved pUC1918 (Schweizer, 1993a). (B) The initial stages of construction of pX1918GT were the same as those of pX1918G. However, the source of the GmR determinant used in this study was pACDGm. This plasmid was constructed in several steps: (i) the unique AseI site found in the promoter region of pACYC184 (Chang and Cohen, 1978) was destroyed by treatment with PolTk, followed by self-ligation. In the resulting pACYC184ΔAse, expression of tet was unaffected; (ii) the 2082-bp EcoRI Ω fragment from pHP45Ω (Prentki et al., 1991) was subcloned into the unique EcoRI site of pACYC184 to form pACΩ; (iii) pACDGm was obtained by subcloning a 830-bp T4 DNA Pol-treated SacI fragment containing the aacC1-encoded GmR determinant from pUCGM (GenBank accession No. U04610) between the AseI sites of pACYC184ΔAse. This procedure flanked aacC1 with the T4 transcription termination signals of the original Ω element since AseI cleaves immediately preceding these signals. Plasmid pALX5 was derived from pALX3 by isolating a 976-bp PolTk-treated, GmR-conferring HindIII fragment from pACDGm and ligation into the unique Scal site located downstream from xylE. Finally, pX1918GT was obtained by excision of a 2417-bp EcoRI fragment fragment from pALX5 and ligating it into EcoRI-cleaved pUC1918 (Schweizer, 1993a). To facilitate nt sequence analysis of fusion junctions generated with the xylE-GmR cassette, the PX1 primer 5'-TTCGCCGATCACGGTCATCG (Tm 59°C), complementary to nt 495–514 of pX1918G and pX1918GT, was designed.
(c) Construction of selectable \textit{xylE} reporter gene cassettes

The aim was to construct cassettes which would contain the selectable \textit{Gm}^R marker downstream from the \textit{xylE} reporter gene. To facilitate fusion analyses of polycistronic operons, two types of cassettes were constructed. One cassette, contained on pX1918G, is a non-polar element, since it contains a \textit{Gm}^R marker without transcriptional terminators, thus allowing transcription of downstream sequences from the \textit{aacC1} promoter. The second cassette, contained on pX1918GT, contains a \textit{Gm}^R marker which is flanked, in inverse orientation, by the T4 transcriptional terminators from the \textit{f\textsubscript{\textalpha}} element (Prentki et al., 1991) which will terminate any transcription initiated within the target DNA sequences or \textit{xylE-Gm}^R, e.g., the \textit{aacC1} promoter. The construction of pX1918G was achieved in several steps as described in Fig. 3. First, after introduction of a unique \textit{SmaI} site into the 3' \textit{xylE} non-coding region on pX1918 (Schweizer, 1993a), a \textit{Gm}^R-containing fragment from pUCGM was cloned into this site. Next, \textit{xylE} and the \textit{Gm}^R determinant were excised on an \textit{EcoRI} fragment from a derivative in which \textit{Gm}^R was in the same transcriptional orientation as \textit{xylE} and recloned into pUC1918 (Schweizer, 1993a). Plasmid pX1918GT was obtained in a similar fashion except that the \textit{Gm}^R determinant was excised from pAC22Gm. This generated a cassette which is flanked, in inverse order, with the restriction sites of the pUC19 (Yanisch-Perron et al., 1985) polylinker. Since in both plasmids most of these sites are unique, the cassettes can be isolated with great ease and the availability of both blunt-ended as well as sticky-ended restriction enzyme-generated termini greatly facilitates their insertion into target DNA. Perhaps the most versatile cassettes are liberated by digestion with \textit{SmaI}, as they can be cloned into any blunt-ended restriction site. The cassettes possess a single, asymmetrically located \textit{BglII} site which can be utilized to determine their orientation in \textit{in vitro} mutagenized DNA by restriction mapping. In addition, the nt sequence of the fusion junction can be determined by double-stranded nt sequencing after priming with the newly designed PX1 primer (see legend to Fig. 3).

(d) Mutagenesis of the \textit{Pa glpD} gene

To test the functionality of both the pEX100T replacement vector as well as the \textit{xylE-Gm}^R fusion cassette, a \textit{glpD}^\textit{--}--\textit{xylE} gene fusion was constructed by insertion of the blunt-ended \textit{xylE-Gm}^R cassette from pX1918GT into the \textit{glpD} gene, which encodes a glycerol- and G3P-inducible G3PDH (Schweizer and Po, 1994). The plasmid-borne fusion was then incorporated into the \textit{Pa} chromosome as described in Fig. 4. The resulting mutants (PAO-XG) did not grow on glycerol but grew normally on all other carbon sources tested. Successful gene replacement was monitored by Southern analysis of chromosomal DNAs (Fig. 5). When the 2.42-kb \textit{EcoRI} \textit{xylE-Gm}^R fragment was used as the probe (panel C, probe 1), the expected 4.66-kb \textit{BsiCI-NcoI} fragment was observed in both mutants analyzed (lanes 2 and 3) which was absent from the wt strain PAO1 (lane 1) (the 1.6-kb weak hybridization signal observed in all lanes is probably due to homology of \textit{xylE} with chromosomal \textit{btzE} sequences of \textit{Pa} (GenBank accession No. X60740)). Reprobing of

![Fig. 4. Gene replacement at the \textit{glpD} locus of \textit{Pa} strain PAO1. Step 1 illustrates the integration of a conjugally transferred \textit{glpD}^\textit{--}--\textit{xylE} fusion plasmid into the homologous chromosomal region of wt strain PAO1 via recombination. The resulting merodiploid strain is \textit{Cb}^R, \textit{Gm}^R and \textit{Suc}^R. Step 2 depicts the excision of unwanted DNA sequences promoted by selecting \textit{Suc}^S in the presence of \textit{Gm} via homologous recombination. In the resulting strain, the \textit{wt glpD} gene is inactivated by the \textit{Gm}^R-marked \textit{glpD}^\textit{--}--\textit{xylE} fusion. Methods: The \textit{glpD} region from PAO1 was isolated from pEB22AE1 (Schweizer and Po, 1994) on a 2248-bp PolII blunt ended \textit{BsiCI-NcoI} fragment and ligated into the \textit{SmaI} site of pUCP18 (Schweizer, 1991b) to form pPS209. Plasmid pPS209 was digested with \textit{BamHI} + \textit{KpnI} and, after treatment with T4 DNA Pol, a 2248-bp fragment was gel-purified and ligated into the \textit{SmaI} site of pEX100T. A plasmid (pPS333) in which the \textit{glpD} was transcribed from \textit{lacz} was retained. Next, the \textit{glpD}^\textit{--}--\textit{xylE} fusion plasmid pPS420 was constructed by (i) digestion of pPS333 with \textit{NotI}, followed by \textit{PolII} treatment, and (ii) ligation with the 2419-bp PolII-treated \textit{xylE-Gm}^R containing \textit{EcoRI} fragment from pX1918GT. Colonies containing \textit{glpD} and \textit{xylE} in the same transcriptional orientation, i.e., expression of C3O from \textit{lacz}, were detected by formation of yellow color after toothpicking a portion of an IPTG-induced colony into 0.5 ml 0.1 M K-phosphate (pH 7.5) containing 100 mM catechol. One representative plasmid was transformed into the mobilizer strain SM10 by a modified CaCl\textsubscript{2} procedure and conjugally transferred into PAO1 as previously described (Schweizer, 1992). Exconjugants were plated on PIA medium containing 300 mg/ml Gm and colonies which appeared on this medium after 24-48-h incubation at 37°C were purified for single colonies on LB + GM plates. Single colonies were then struck on LB medium containing 5% Suc. After overnight incubation at 37°C, Suc^R colonies were obtained at high frequencies and the majority of these were \textit{Cb} R, indicating that they had lost the pEX100T vector-associated sequences. Alternatively, the conjugation mixtures may be plated directly on selective medium containing 5% sucrose before testing for \textit{Cb} R.)
induced cells (Table I). As expected, C230 was synthesized in PAO-XG cells but not in PAO1 cells. In addition, C230 synthesis in PAO-XG was inducible by addition of both glycerol and G3P to the growth medium.

(e) Conclusions

(1) A gene replacement vector, pEX100T, was developed which was genetically engineered to facilitate the cloning and genetic manipulation of virtually any DNA fragment. This was achieved by incorporating into a single vector (i) the counterselectable sacB marker; (ii) a lacZα allele for blue-white screening; (iii) an oriT for conjugation-mediated plasmid transfer and (iv) unique cloning sites for SmaI and the rare-cutting meganuclease I-SceI. By site-specific mutagenesis and subcloning, most restriction sites within the replacement vector were eliminated to give greater access to restriction sites within the cloned fragment, thus facilitating the construction of marked or unmarked insertions, deletions and frameshifts. The ApR marker contained on this plasmid can easily be replaced by cloning other antibiotic resistance-encoding gene cassettes into a unique ScaI site within bla. Despite being based on the high-copy number vector pUC19Sce, pEX100T is stable in our hands. This vector has been successfully used by us and others to construct various mutants in Pa. As with all sacB-bearing plasmids it is imperative to monitor their Suc5 phenotype at each step during plasmid constructions and transformations. This is best done by streaking single colonies on Suc-containing medium. If this phenotype is not checked mutations leading to spontaneous Suc5 may accumulate in the process.

(2) The replacement vector pEX100T is based on the helper plasmid pUC19Sce. This plasmid contains the same rare restriction sites as pEX100T within lacZα. Thus, in the unlikely event in which the restriction sites

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**Fig. 5. Genomic Southern analysis of Pa glpD'·xylE fusion strains.** (A) and (B) Physical maps of the glpD regions of wt PAO1 and glpD·xylE-GmR mutants, respectively. (C) Southern analysis. Lanes: 1, DNA from wt PAO1; 2 and 3, DNAs from PAO1 strains containing a glpD'·xylE fusion inactivating the wt glpD locus. Dots mark fragments weakly hybridizing to probe 1 and the arrowhead marks the 2.24-kb BsiCIdigested fragment of wt PAO1. Biotinylated DNA size markers (λ/HindIII and 4X174/HaeII fragments) in kb are indicated at the right. **Methods:** Chromosomal DNA from PAO1 and from two PAO1 strains harboring glpD'·xylE fusions were isolated similarly to the procedure of Meade et al. (1982) described for Rhizobium. BsiCIdigested chromosomal DNA fragments were electophoresed on a 1% agarose gel in TAE buffer and were transferred to Photogene nylon membranes (Gibco BRL) as previously described (Sambrook et al., 1989). Two fragments were used as the probe. The 2.42-kb EcoRI fragment from pACO26 containing xylE and GmR was used as DNA probe 1. The same blot was stripped of probe 1 and reprobed with the 728-bp PstI fragment from glpD locus. This was achieved by incorporating into a single vector (i) the counterselectable sacB marker; (ii) a lacZα allele for blue-white screening; (iii) an oriT for conjugation-mediated plasmid transfer and (iv) unique cloning sites for SmaI and the rare-cutting meganuclease I-SceI. By site-specific mutagenesis and subcloning, most restriction sites within the replacement vector were eliminated to give greater access to restriction sites within the cloned fragment, thus facilitating the construction of marked or unmarked insertions, deletions and frameshifts. The ApR marker contained on this plasmid can easily be replaced by cloning other antibiotic resistance-encoding gene cassettes into a unique ScaI site within bla. Despite being based on the high-copy number vector pUC19Sce, pEX100T is stable in our hands. This vector has been successfully used by us and others to construct various mutants in Pa. As with all sacB-bearing plasmids it is imperative to monitor their Suc5 phenotype at each step during plasmid constructions and transformations. This is best done by streaking single colonies on Suc-containing medium. If this phenotype is not checked mutations leading to spontaneous Suc5 may accumulate in the process.
still present in the new vector interfere with the intended cloning experiments pUCScE may serve as an auxiliary plasmid from which the manipulated DNA can be excised by I-SceI digestion without fear of inactivation of cloned DNA segments. I-SceI recognizes a non-symmetric 18-nt sequence which to this date has not been found in bacterial nt sequences.

(2) Two selectable xylE fusion cassettes were developed which facilitate the construction of plasmid-borne xylE fusions and their subsequent single-copy incorporation into the bacterial chromosome. The availability of cassettes with and without transcriptional terminators facilitates fusion analyses of polycistronic operons. The presence of the IPTG-inducible lacZpo on pEX100T allows fusion construction to promoter-less genes or gene fragments by (i) cloning the target sequences in the same transcriptional orientation as lacZpo and (ii) screening for IPTG-inducible C23O synthesis in lacI*-containing host strains. Subsequent homologous recombination with the host chromosome will then place xylE transcription under the control of the endogenous regulatory sequences.

(4) The functionality of pEX100T and the xylE-GmR cassette was demonstrated by construction of a plasmid-borne gpd::xylE-GmR insertion and its subsequent integration into and regulated expression from the Pa chromosome. Although not explicitly explored in this study, the tools described here should be widely applicable to other pathogenic and non-pathogenic bacteria provided that (i) they do not support replication of pMB1-based plasmids, (ii) they are recipients of oriT-mediated conjugation and (iii) they are SucS upon expression of sacB.

ACKNOWLEDGEMENTS

This work was supported by grants from the Canadian Cystic Fibrosis Foundation and the Medical Research Council of Canada (MT-11245). H.P.S. is a MRC Medical Scholar. We thank M. Bacic for pPS333 and assistance with the C23O assays, and T. de Kievit, University of Guelph, for communicating unpublished results.

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