

# Generation of transposon insertion mutant libraries for Gram-positive bacteria by electroporation of phage Mu DNA transposition complexes

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Transposon mutagenesis is a powerful technique for generating collections of insertion mutants for genetic studies. This paper describes how phage Mu DNA transposition complexes, transpososomes, can be exploited for gene delivery to efficiently introduce selectable markers to genomes of Gram-positive bacteria. Mu transpososomes were assembled *in vitro* with custom-designed mini-Mu transposons, concentrated, and electroporated into cells of three Gram-positive bacterial species: *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus suis*. Within cells, the complexes reproduced an authentic DNA transposition reaction and integrated the delivered transposons into the bacterial genomes, yielding single-copy insertions. The integration efficiency among different species and strains of Gram-positive bacteria ranged from  $1 \times 10^1$  to  $2 \times 10^4$  c.f.u. ( $\mu\text{g}$  introduced transposon DNA)<sup>-1</sup>. The strategy should be applicable to a variety of other Gram-positive species after initial optimization of certain key factors affecting transposon delivery, such as the preparation method of competent cells and physical parameters of electroporation. This study extends the scope of the Mu transpososome delivery-based genomic DNA integration strategy to Gram-positive bacteria. Thus, a straightforward generation of sizeable mutant banks is feasible for these bacteria, potentiating several types of genomic-level approaches for studies of a variety of important bacterial processes, such as pathogenicity.

Received 3 December 2004  
Revised 4 January 2005  
Accepted 17 January 2005

## INTRODUCTION

Rapidly evolving techniques and strategies of bacterial genetics/genomics constitute powerful means to dissect diverse mechanisms involved in a variety of microbial functions (Boeke, 2002; McDevitt & Rosenberg, 2001). At present, the most efficient strategies typically involve the generation of genomic insertion mutant libraries and characterization of phenotypic properties of individual clones within the libraries, preferably in a setting in which it is possible to study a large number of mutants simultaneously (Hamer *et al.*, 2001). A studied pool may be an ordered collection of insertion mutants, each mutant containing a defined mutation in a particular nonessential gene (Judson & Mekalanos, 2000), or it may be a random collection of insertion mutants (Berg & Berg, 1996). While the generation of ordered collections is extremely laborious and time-consuming, and in many cases non-applicable, transposon insertion mutagenesis strategies provide straightforward means to generate random insertion libraries for a

number of bacterial species (Berg & Berg, 1996; Boeke, 2002).

Transposons have been exploited as genetic tools in a variety of organisms (Craig *et al.*, 2002). Although most transposons operate only *in vivo*, several of the more advanced transposon-based insertion mutagenesis systems are functional also *in vitro* (Boeke, 2002). Furthermore, a combined *in vitro* and *in vivo* approach is currently feasible with two of the systems: Tn5 and Mu. With both of these transposons, the strategy involves an initial assembly of the transposition machinery *in vitro*, subsequent introduction of such machineries into recipient cells, and genomic integration of the delivered transposon DNA within these cells (Goryshin *et al.*, 2000; Lamberg *et al.*, 2002).

Bacteriophage Mu transposition is one of the best-characterized transposition systems (Chaconas & Harshey, 2002). In the simplest *in vitro* set-up, Mu transposition into an intermolecular target requires MuA transposase, transposon DNA and target DNA as the only macromolecular components (Haapa *et al.*, 1999a). This minimal-component reaction proceeds via initial *in vitro* assembly

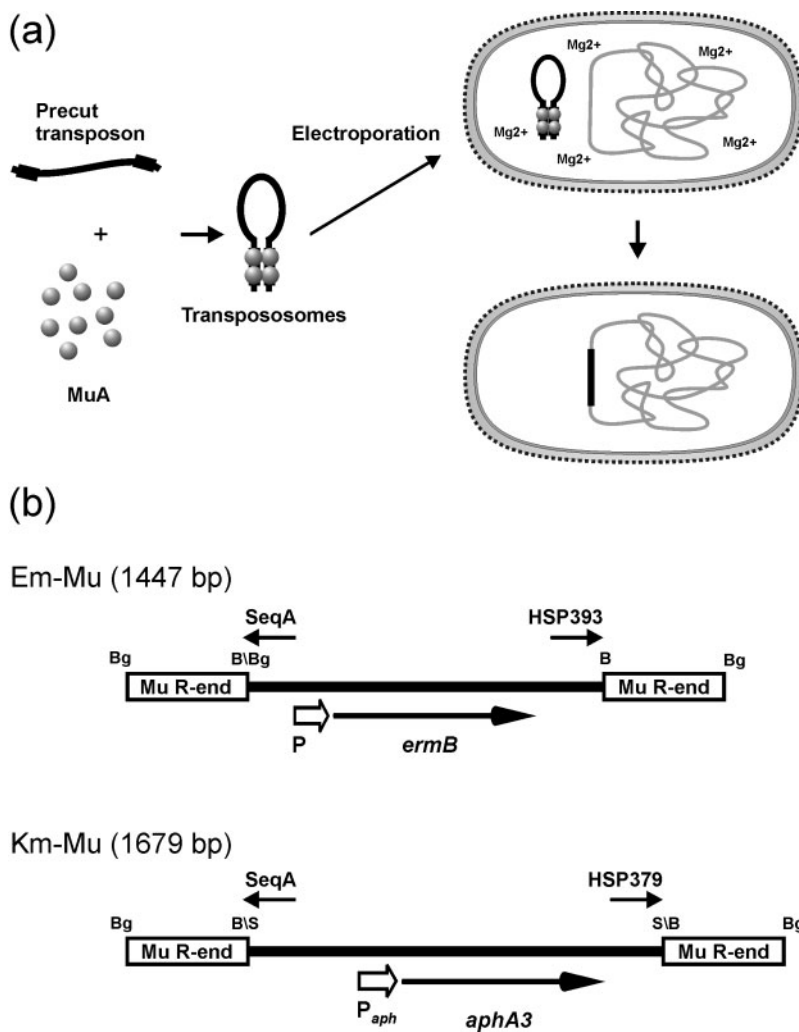
Abbreviation: R-end, right-end.

of a stable nucleoprotein complex, the Mu transpososome, which is catalytically inactive in the absence of divalent cations, but becomes activated for transposition chemistry in the presence of divalent cations such as  $Mg^{2+}$  (Savilahti *et al.*, 1995). Similarly, this activation can occur within bacterial cells following electroporation of assembled complexes, thus facilitating transposon integration into host chromosomal DNA (Fig. 1a), as demonstrated with Gram-negative bacteria (Lamberg *et al.*, 2002).

Many important human and animal pathogens belong to the Gram-positive staphylococci or streptococci. *Staphylococcus aureus* remains a frequent cause of community-acquired and nosocomial infections ranging from minor skin ailments to life-threatening deep-tissue infections (Lowy, 1998). The morbidity and mortality from *Staph. aureus* infections are high because of the intrinsic virulence of the species: its ability to cause a diverse array of life-threatening infections and its ability to adapt to different host environments (Proctor, 2000). *Streptococcus pyogenes* causes a wide variety of human diseases, such as pharyngitis, impetigo, erysipelas, necrotizing fasciitis

and toxic-shock-like syndrome (Chelsom *et al.*, 1994). *Streptococcus suis* is an important pig pathogen that causes severe infections, such as sepsis and meningitis, and occasionally it is life-threatening to humans (Staats *et al.*, 1997).

Most of the transposition mutagenesis strategies currently in use for Gram-positive bacteria are based on transposon mobilization *in vivo*. Typically, these strategies may involve conjugative transposons (Scott, 1993), or they may utilize transposon delivery systems that employ temperature-sensitive replication-deficient vector plasmids (Youngman, 1993). Transposons Tn551, Tn917 and Tn916 have traditionally been used for insertional mutagenesis in staphylococci and streptococci, and these elements have proven valuable in gene identification in these organisms (Caparon & Scott, 1991; Mei *et al.*, 1997; Novick, 1991; Slater *et al.*, 2003). Similarly, *Himar1*-based transposons, as well as a derivative of Tn4001, have recently been utilized for efficient mutagenesis of staphylococci and streptococci (Bae *et al.*, 2004; Barnett & Scott, 2002; Lyon *et al.*, 1998; May *et al.*, 2004).



**Fig. 1.** (a) *In vivo* integration of *in vitro* assembled Mu transpososomes. A tetramer of MuA transposase and mini-Mu transposon ends assemble into a stable protein-DNA complex. Upon encountering  $Mg^{2+}$  ions *in vivo*, the complex executes a random two-ended integration of transposon DNA into the chromosome. (b) DNA substrates used in this study. Linear mini-Mu transposons in this study are defined as segments of DNA that contain 50 bp Mu R-end DNA as inverted repeats at each end (Haapa *et al.*, 1999a; Savilahti *et al.*, 1995). Em-Mu and Km-Mu mini-Mu transposons were released from their carrier plasmids by *Bgl*I digestion, which leaves four-nucleotide 5' overhangs flanking the exposed transposon ends. In this pre-cut form, the transposon 3' ends are exposed and readily available for the transposon integration reaction, also known as a strand transfer reaction (Craigie & Mizuuchi, 1987; Haapa *et al.*, 1999a; Savilahti *et al.*, 1995). *ermB* and *aph3*, adenine methylase and aminoglycoside 3' phosphotransferase genes, respectively. Small arrows above the transposons indicate the binding sites of primers used for DNA sequencing. Restriction sites: Bg, *Bgl*I; B, *Bam*HI; S, *Sal*I. Cloning joints (see Methods) are indicated, with B\Bg, B\S and S\B referring to the combination of the restriction sites used.

Genomic-level approaches are needed for advanced studies on important bacterial processes such as pathogenicity, and many of these studies require the generation of large mutant collections. In this communication, we describe a general gene delivery strategy for introducing selectable markers into Gram-positive bacteria for the benefit of genomic studies, and discuss the merits and limitations of this DNA transposition-based strategy with respect to the most important technical variables. The methodology should be applicable to any bacterial species given that reasonable electroporation efficiencies can be achieved.

## METHODS

**Bacterial strains and culture conditions.** *Escherichia coli* laboratory strain DH10B (Grant *et al.*, 1990) was used for routine plasmid DNA isolation and as a standard cloning host. Wild-type *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogenes* A8173 (Hytönen *et al.*, 2000) and *Streptococcus suis* D282 (Vecht *et al.*, 1989) were from the laboratory collection of J.F. at the University of Turku. *Staph. aureus* S30 was from William Kelley and Jacques Schrenzel, University of Geneva Hospital, Geneva, Switzerland. Control replicative plasmids for electroporation, pSK265 and pLZ12-Km (see Table 1), were isolated from *Staph. aureus* 8325-4 (Novick, 1967) and *E. coli* DH1 (Hanahan, 1983), respectively. All bacteria were grown at 37 °C. For *E. coli* cultures, standard conditions with Luria–Bertani medium were used (Sambrook & Russell, 2001). *Staph. aureus* strains were cultured using brain heart infusion (BHI) medium (Difco). *Strep. suis* and *Strep. pyogenes* strains were cultured using THY medium, which consists of Todd–Hewitt broth (Difco) supplemented with 0.5% (w/v) yeast extract (Difco). For culture dishes, media were solidified by the addition of 1.5% Bacto agar (Difco). Liquid cultures of Gram-positive bacteria were grown using gentle agitation (100 r.p.m.), and on solid media, these bacteria were grown under anaerobic conditions produced by a gas generator (BBL GasPakPlus; BD Biosciences). For storage, each bacterial strain was frozen as a glycerol stock suspension at –80 °C in its respective growth medium containing 15% (v/v) glycerol. When appropriate, plasmid maintenance and genomic transposon insertions were

selected using antibiotics (Sigma) in the growth media at the following concentrations: *E. coli*, 100 µg ampicillin (Ap) ml<sup>-1</sup>, 100 µg erythromycin (Em) ml<sup>-1</sup>, 30 µg kanamycin (Km) ml<sup>-1</sup>; *Staph. aureus*, 10 µg Em ml<sup>-1</sup>, 10 µg chloramphenicol (Cm) ml<sup>-1</sup>; *Strep. pyogenes*, 1 µg Em ml<sup>-1</sup>, 500 µg Km ml<sup>-1</sup>; *Strep. suis*, 500 µg Km ml<sup>-1</sup>.

**Proteins and reagents.** Restriction endonucleases, Klenow enzyme and T4 DNA ligase were from New England Biolabs, RNase A was from Roche, and lysostaphin was from Ambi Products LLC. Calf intestinal phosphatase, proteinase K, DyNAzyme II DNA polymerase and MuA transposase (MuA) were from Finnzymes. Lysozyme, BSA, Ficoll 400 and heparin were from Sigma. Triton X-100 was from Fluka, and [ $\alpha$ -<sup>32</sup>P]dCTP (1000–3000 Ci mmol<sup>-1</sup>; 37 000–111 000 GBq mmol<sup>-1</sup>) was from Amersham. Agaroses (NuSieve 3:1 and Seakem GTG) were from Cambrex.

**Standard DNA techniques.** Plasmid DNA from *E. coli* and *Staph. aureus* was isolated using purification kits from Qiagen, as recommended by the supplier, with the exception that collected *Staph. aureus* cells were resuspended in 300 µl PBS (Sambrook & Russell, 2001), and treated with lysostaphin (200 µg ml<sup>-1</sup>) at 37 °C for 15 min prior to plasmid extraction. Standard DNA manipulation and cloning techniques, including PCR for plasmid engineering, were performed as described by Sambrook & Russell (2001), and DNA-modifying enzymes were used as recommended by the suppliers. DNA sequence determination was performed at the DNA sequencing facility of the Institute of Biotechnology (University of Helsinki) by using the BigDye terminator cycle sequencing kit and the ABI 377 XL sequencer, both from Applied Biosystems.

**Chromosomal DNA isolation.** Chromosomal DNA from Gram-positive bacteria was isolated as follows. Cells from an overnight culture (5 ml) were collected by centrifugation and resuspended in 200 µl lysis buffer (6.7% sucrose; 50 mM Tris/HCl, pH 7.0; 1 mM EDTA). RNase A and lysozyme (4 µl each from 10 mg ml<sup>-1</sup> and 100 mg ml<sup>-1</sup> stock solutions, respectively) were added to the suspension, which was then incubated at 37 °C for 30 min. Subsequently, 8 µl 20 mg proteinase K ml<sup>-1</sup> and 5 µl 20% SDS were added, and the suspension was incubated at 56 °C for 30 min. The suspension was then extracted once with phenol, once with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), and once with chloroform/isoamyl alcohol (24:1, v/v). DNA was ethanol-precipitated from the

**Table 1.** Antibiotic-resistant colonies detected following electroporation into different bacterial strains

Substrate DNA	Resistance to	Amount electroporated (ng)	Pre-incubation with MuA	Antibiotic-resistant colonies [c.f.u. (µg DNA) <sup>-1</sup> ]			
				<i>Staph. aureus</i> *		<i>Strep. pyogenes</i>	<i>Strep. suis</i> †
				ATCC 29213	S30	A8173	D282
Em-Mu	Em	800	Yes	2 × 10 <sup>4</sup>	1.2 × 10 <sup>3</sup>	3 × 10 <sup>1</sup>	ND
Em-Mu	Em	500	No	–	–	–	ND
Km-Mu	Km	800	Yes	–	ND	1 × 10 <sup>1</sup>	1 × 10 <sup>2</sup>
Km-Mu	Km	500	No	–	ND	–	–
pSK265‡	Cm	5	No	4 × 10 <sup>6</sup>	5.6 × 10 <sup>5</sup>	–	–
pLZ12-Km§	Km	100	No	–	ND	5 × 10 <sup>4</sup>	2 × 10 <sup>5</sup>

ND, Not determined.

–, No colonies detected.

\*The Km<sup>R</sup> cassette does not confer resistance to Km in *Staph. aureus*, even when present within a multicopy plasmid.

†*Strep. suis* is naturally resistant to Em.

‡Replicative control plasmid isolated from *Staph. aureus* 8325-4.

§Replicative control plasmid isolated from *E. coli* DH1.

water phase, and resuspended in 50  $\mu$ l TE buffer (10 mM Tris/HCl, pH 7.5; 0.5 mM EDTA). DNA yields from standard 5 ml cultures ranged between 10 and 50  $\mu$ g.

**Plasmids.** Plasmid pUC19 was from New England Biolabs. Plasmid pLZ12-Km (Hanski *et al.*, 1992) is an *E. coli*-*Streptococcus* sp. shuttle vector that includes a gene (*aphA3*) encoding Km resistance, and it was obtained from Michael Caparon, Washington University, St Louis, MO, USA. *Staphylococcus* sp. plasmid pSK265 (Jones & Khan, 1986) includes a gene (*cat*) encoding Cm resistance, and it was obtained from William Kelley. Plasmid pLEB21 (Qiao *et al.*, 1996) was obtained from Per Saris, University of Helsinki; this plasmid is a modified pUC6S (Vieira & Messing, 1991) in which a cloned *Lactobacillus reuteri* gene (*ermB*) encoding Em resistance (Axelsson *et al.*, 1988) is inserted at a *Sma*I site. Plasmid pVTF1 is a derivative of plasmid pEntranceposon-Kan<sup>R</sup> (F-766; Finnzymes), and it was obtained from Ville Tieaho, Finnzymes. Instead of the entire vector backbone between two *Bgl*II sites, pVTF1 contains a pUC19 origin of replication (nucleotides 848–1571 of pUC19, numbered as indicated by New England Biolabs). Furthermore, an additional *Bgl*II site has been engineered into this origin at position 1197.

**Mini-Mu transposons.** The pUC19-derived carrier plasmids pLEB620 and pPTH2 for transposons Em-Mu and Km-Mu, respectively (Fig. 1b), were constructed as follows. The 1.27 kb *Bam*HI/*Bgl*II fragment of pLEB21 that includes the *ermB* gene was ligated to the 0.92 kb *Bam*HI fragment of pVTF1 to yield pLEB620. Similarly, the 1.5 kb *Sal*I fragment of pLZ12-Km that includes the *aphA3* gene was end-filled with Klenow enzyme and ligated to the end-filled 0.92 kb *Bam*HI fragment of pVTF1 to yield pPTH2. To confirm authenticity, these two plasmids were sequenced by using the Mu *in vitro* transposition-based DNA sequencing kit (Template Generation System, Finnzymes). From their respective carrier plasmids, mini-Mu transposons were released by *Bgl*II digestion that leaves four-nucleotide 5' overhangs flanking the exposed transposon ends. Such an end configuration in mini-Mu transposons ensures efficient assembly of stable transpososomes (Haapa *et al.*, 1999b; Savilahti *et al.*, 1995). DNA fragments were purified using anion-exchange chromatography, as described by Haapa *et al.* (1999b).

#### Electrocompetent cells

Electrocompetent cells for *E. coli* were prepared, stored and used as described previously (Lamberg *et al.*, 2002). Electrocompetent cells for Gram-positive bacterial strains were prepared as described below. These preparations were used either directly (fresh) for electroporation, or they were frozen as aliquots in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until thawed for electroporation. The viable counts of these preparations were routinely  $\sim 5 \times 10^{10}$  c.f.u. ml<sup>-1</sup>. Approximately 70% of the cells survived the freezing step.

***Staph. aureus.*** An overnight culture in THY medium was diluted (1:500) in a total volume of 200 ml THY and grown at  $37^{\circ}\text{C}$  with gentle agitation to an OD<sub>600</sub> of  $\sim 0.4$ . Cells were collected by centrifugation at 3000 r.p.m. (1248 g) in a Heraeus Biofuge Primo fixed-angle rotor at  $4^{\circ}\text{C}$  for 15 min. Cells were then washed by resuspension in 20 ml ice-cold 0.5 M sucrose and collected by subsequent centrifugation as above. This washing step was repeated. The cells were then resuspended in 10 ml ice-cold solution containing 0.5 M sucrose and 10% (v/v) glycerol, and sedimented again by centrifugation, as described above. Subsequently, the cell pellet was resuspended in 0.5 M sucrose and 10% glycerol, in a total volume of 0.5 ml.

***Strep. suis.*** Cells were grown in THY medium supplemented with 30 mM glycine to an OD<sub>600</sub> of  $\sim 0.2$ . Subsequently, the cells were prepared as described previously (Pulliainen *et al.*, 2003).

***Strep. pyogenes.*** Cells were grown in THY medium to an OD<sub>600</sub> of  $\sim 0.3$ . Subsequently, the cells were prepared as described previously for *Strep. suis* (Pulliainen *et al.*, 2003).

**Transpososome assembly.** The *in vitro* transpososome assembly was performed as described previously (Lamberg *et al.*, 2002). The standard assembly reaction (40  $\mu$ l) contained 2.2 pmol ( $\sim 2.0$   $\mu$ g) transposon DNA, 9.8 pmol (0.8  $\mu$ g) MuA, 150 mM Tris/HCl (pH 6.0), 50% (v/v) glycerol, 0.025% (w/v) Triton X-100, 150 mM NaCl and 0.1 mM EDTA. The reaction was carried out at  $30^{\circ}\text{C}$  for 2 h, and the assembly of transpososomes was monitored by agarose (NuSieve 3:1) gels containing BSA (87  $\mu$ g ml<sup>-1</sup>) and heparin (87  $\mu$ g ml<sup>-1</sup>), as described previously (Lamberg *et al.*, 2002).

#### Concentration of transpososomes and electroporation.

Transpososomes were concentrated, and the preparation was desalted for electroporation as follows. Initially, a total of 16 standard assembly reactions were pooled, and the pool volume was brought to 4 ml with water. The mixture was then filtered using a Centricon YM-100 centrifugal cartridge (100 kDa cut-off; Millipore), according to the manufacturer's instructions. The retentate was then desalted by passage with 2 ml water, yielding a final transpososome stock for electroporation. An approximate tenfold increase in transpososome concentration was achieved by this method, as estimated from the final volume of the stock ( $\sim 60$   $\mu$ l).

For electroporation, thawed electrocompetent cells (50  $\mu$ l) were initially mixed on ice with 1 or 2  $\mu$ l transpososome preparation; transposon preparations without the addition of MuA and replicative plasmid preparations were used in control experiments. The mixture was next transferred to a pre-chilled 0.1 cm electrode spacing cuvette (Bio-Rad). Electroporation was then performed using a Gene Pulser II electroporator (Bio-Rad) with the following settings: *Staph. aureus* (100  $\Omega$ , 2.3 kV, 25  $\mu$ F), *Strep. pyogenes* and *Strep. suis* (200  $\Omega$ , 1.8 kV, 15  $\mu$ F). Subsequently, 1 ml pre-warmed THY medium supplemented with 0.3 M sucrose was added, and the suspension was incubated at  $37^{\circ}\text{C}$  for 90 min with gentle agitation (*Staph. aureus*) or 2 h without agitation (*Strep. pyogenes* and *Strep. suis*). For the selection of genomic integrations, the cells were then spread on agar plates containing BHI (*Staph. aureus*) or THY (*Strep. pyogenes* and *Strep. suis*), and appropriate antibiotics. Alternatively, glycerol (100%) was added to the cell suspension to the final concentration of 15% (v/v), the suspension was frozen as aliquots in liquid nitrogen, and the aliquots were stored at  $-80^{\circ}\text{C}$  until thawed for selection as above.

**Southern blotting.** For blotting, 2.5  $\mu$ g chromosomal DNA was digested with *Pvu*II and separated on a 0.8% Seakem GTG agarose gel. The DNA was transferred with 0.4 M NaOH to a nylon filter (Hybond-N+, Amersham) and fixed with UV light (Stratalinker UV cross-linker; Stratagene). Southern hybridization was performed essentially as described by Sambrook & Russell (2001), with  $\alpha$ -<sup>32</sup>P-labelled transposon (Em-Mu or Km-Mu) probes (Random Primed, Roche). Visualization was done by autoradiography using the Fujifilm Image Reader BAS-1500.

**Determination of chromosomal target sites.** The chromosomal DNA of each antibiotic-resistant isolate was digested as follows: with *Bam*HI for Km-Mu isolates of *Strep. suis* and *Strep. pyogenes*, with *Pvu*II for Em-Mu isolates of *Strep. pyogenes* and *Staph. aureus* ATCC 29213, and with *Nhe*I + *Spe*I for Em-Mu isolates of *Staph. aureus* S30. In each case, the digestion generated a DNA fragment with an intact transposon attached to its flanking chromosomal DNA. These fragments were then cloned into the *Bam*HI, *Sma*I or *Xba*I site of pUC19, respectively, using appropriate selection schemes with two antibiotics. DNA sequences of transposon borders were determined from these plasmids using the following primers that read the sequence outwards from within the transposons as specified in

**Table 2.** Transposon integration sites and target site duplications

Strain	Clone	Sequence*	Transposon orientation		Genomic location§	Genome		
			Genome†	ORF‡				
<i>Staph. aureus</i>	SE1	tagggaatcc <b>ACCCA</b> (Em-Mu) <b>ACCCA</b> aatgtttact	-	+	Malate:quinone oxidoreductase	2689796–2689800	N315	
	SE2	taaaaaaggt <b>ATGTT</b> (Em-Mu) <b>ATGTT</b> attagtaact	+	+	Hypothetical protein	664009–664013	N315	
	ATCC 29213	cgtagcttt <b>ACTAC</b> (Em-Mu) <b>ACTAC</b> caagataat	-	+	Transcription anti-terminator BglG	377991–377995	N315	
	SE5	atttcattaa <b>CTAAC</b> (Em-Mu) <b>CTAAC</b> cttaactgttg	+	+	Intergenic (in pathogenicity island 2)	446721–446725	N315	
	SE7	actaagtacc <b>ATATG</b> (Em-Mu) <b>ATATG</b> atcaatggca	+	+	Conserved hypothetical protein	782041–782045	N315	
	SE8	atttcacagt <b>ACAGT</b> (Em-Mu) <b>ACAGT</b> agatacaggg	-	+	Ornithine aminotransferase	209600–209604	N315	
	SE10	aaaatagtca <b>TTGAT</b> (Em-Mu) <b>TTGAT</b> tattattgga	+	+	ABC transporter ATP-binding protein	741621–741625	MRSA252	
	SE15	gcagtgagag <b>GTGTA</b> (Em-Mu) <b>GTGTA</b> gattttatt	+	+	ATP-binding protein OppD	960107–960111	N315	
	<i>Staph. aureus</i>	G1	tttaaaata <b>AAGAA</b> (Em-Mu) <b>AAGAA</b> actgctatg	+	+	Accessory gene regulator A, AgrA	2082160–2082164	N315
		G2	attcatctt <b>CCAGA</b> (Em-Mu) <b>CCAGA</b> tgatctct	-	+	5S rRNA	2233501–2233505	N315
S30		agatggat <b>CCCCG</b> (Em-Mu) <b>CCCCG</b> ctctctttt	+	+	Intergenic (upstream of <i>phpA</i> )	1266343–1266347	N315	
G4		atctcaccat <b>TTTGT</b> (Em-Mu) <b>TTTGT</b> aaagtaata	+	+	ABC transporter	1590795–1590799	N315	
G5		taccacagt <b>CCGTT</b> (Em-Mu) <b>CCGTT</b> gtcacacct	-	+	FmtP protein	2224234–2224238	N315	
G6		tacagtatg <b>GCTGA</b> (Em-Mu) <b>GCTGA</b> agtaacggct	+	+	D-Serine/D-alanine/glycine transporter	1732854–1732858	N315	
G7		gtatttatt <b>ACAAC</b> (Em-Mu) <b>ACAAC</b> ctctattat	-	+	Intergenic (downstream of <i>porA</i> )	1284049–1284053	N315	
G8		aaaatagt <b>TCGGG</b> (Em-Mu) <b>TCGGG</b> tacagtagcg	-	+	Glutaryl-CoA dehydrogenase	271939–271943	N315	
G9		tcagctaat <b>TCGTC</b> (Em-Mu) <b>TCGTC</b> tacattgg	-	+	General stress protein 20U	2191007–2191011	N315	
<i>Strep. pyogenes</i>	A1	gttagttcg <b>TCCTG</b> (Km-Mu) <b>TCCTG</b> ttttgccaaa	+	-	Aldose-1-epimerase	1421601–1421605	M3	
	AE1	tgcccgat <b>ACCAA</b> (Em-Mu) <b>ACCAA</b> gtgtttcaa	+	-	Laminin-binding protein	1674772–1674776	M1	
	A8173	cgaggtttg <b>GTGGT</b> (Em-Mu) <b>GTGGT</b> caactgtaa	-	+	Maltodextrin permease MalC	1078335–1078339	M1	
	AE4	gcgctttg <b>ACCAA</b> (Em-Mu) <b>ACCAA</b> gaacttagg	+	+	Thioredoxin reductase	701627–701631	M1	
	AE10	ttaatattg <b>GGGAA</b> (Em-Mu) <b>GGGAA</b> gtagagact	+	+	SrtB involved in streptolysin S formation	6967–6971	BL-T	
<i>Strep. suis</i>	D1	tagataact <b>CCTAT</b> (Km-Mu) <b>CCTAT</b> ccaagtcac	+	+	Transcription termination protein NusB	NA	P1/7	
	D2	gggcttcac <b>ACCGT</b> (Km-Mu) <b>ACCGT</b> gaaccaggta	+	+	GlcNAc-6-P deacetylase	NA	P1/7	
	D282	cgaggttcg <b>CTCAG</b> (Km-Mu) <b>CTCAG</b> gactaaatc	+	-	Conserved hypothetical protein	NA	P1/7	
	D5	tttgattagt <b>CGAGT</b> (Km-Mu) <b>CGAGT</b> gaaaaatta	+	+	Methionine sulfoxide reductase	NA	P1/7	
	D6	ttctgcat <b>AGGAG</b> (Km-Mu) <b>AGGAG</b> acagtcgagt	+	+	RNA methyl-transferase	NA	P1/7	
	D7	gtgtcatat <b>GCTGA</b> (Km-Mu) <b>GCTGA</b> cataatgctg	-	-	Aminopeptidase P	NA	P1/7	
	D8	tctacgtaag <b>TTTGC</b> (Km-Mu) <b>TTTGC</b> caatccct	+	+	RNA helicase	NA	P1/7	
	D9	ttaacgttc <b>TTTTC</b> (Km-Mu) <b>TTTTC</b> ctgtctctt	-	+	Intergenic (upstream of <i>mrp</i> )	NA	P1/7	
	D10	ggtcgtcac <b>AACAG</b> (Km-Mu) <b>AACAG</b> gaagtcgctt	+	-	Dextran glucosidase DexS	NA	P1/7	

NA, Not applicable; genomic coordinate unknown.

\*Target site duplications are shown in bold capitals.

†Compared with the genomic sequences shown, the transcription from the transposon proceeds from left to right (+) or from right to left (-).

‡Transcription from the transposon compared with the direction of local transcription within the specified genomic location; +, same direction; -, opposite direction.

§Genomic location of *Staph. aureus*, *Strep. pyogenes* and *Strep. suis* insertions was determined by comparison with the following respective data: the complete sequences of *Staph. aureus* N315 (GenBank accession no. NC\_002745; Kuroda *et al.*, 2001) or *Staph. aureus* MRSA252 (NC\_002952; Holden *et al.*, 2004); the complete genomes of *Strep. pyogenes* serotypes M1 (NC\_002737; Ferretti *et al.*, 2001) or M3 (NC\_004070; Beres *et al.*, 2002); and the unfinished sequence of *Strep. suis* P1/7 (NC\_004549; Sanger Institute). Additionally, the genomic location of *Strep. pyogenes* clone AE10 insertion was determined by comparison with the *Strep. pyogenes* BL-T (AB030831; Karaya *et al.*, 2001) lantibiotic streptin gene cluster.

||When applicable, encoded protein or transcribed RNA is indicated.

Fig. 1(b). SeqA, 5'-ATCAGCGGCCGCGATCC-3'; HSP-393, 5'-GAGGTCCTAGCGCCTACG-3'; HSP-379, 5'-GGAATTTGTATCGT-CGATCC-3'. Genomic transposon insertion site loci (Table 2) were identified by comparing the insertion site data to publicly available genomic sequences using BLAST database searches on the National Center for Biotechnology Information and the Wellcome Trust Sanger Institute servers. ORFs were analysed by using web-based programs on the European Bioinformatics Institute server.

## RESULTS

### Mini-Mu transposons

The Mu transpososome delivery-based genomic DNA integration strategy, initially developed for Gram-negative bacteria (Lamberg *et al.*, 2002), is characteristically species

non-specific. Therefore, it was of interest to examine whether a similar strategy would yield genomic integrations in Gram-positive bacteria also. For these studies, we constructed two mini-Mu transposons with suitable properties and utilized three species of Gram-positive bacteria, *Staph. aureus*, *Strep. pyogenes* and *Strep. suis*, as model recipient cells. The transposons (see Methods and Fig. 1b) contain a marker gene selectable in Gram-positive bacteria and, at each end as an inverted repeat, a 50 bp segment of Mu right-end (R-end) sequence, including the critical MuA transposase binding sites that are necessary and sufficient for Mu transpososome assembly (Haapa *et al.*, 1999a; Savilahti *et al.*, 1995). The Em-Mu transposon includes the selectable marker *ermB* and its constitutive promoter from *L. reuteri*, thereby conferring resistance to Em. Similarly, the Km-Mu transposon includes the *aphA3* gene and its control sequences from Tn1545 for selection with Km. These gene cassettes were chosen for genomic mutagenesis because the *ermB* cassette has been used to generate chromosomal knockout mutants in *Lactococcus lactis* (Qiao *et al.*, 1996), and the *aphA3* cassette has been used for signature-tagged mutagenesis in *Listeria monocytogenes* (Autret *et al.*, 2001).

### Assembly and concentration of transpososomes

Mu transpososomes were assembled by incubating Em-Mu and Km-Mu transposons with MuA transposase in the absence of divalent metal ions (see Methods), and transpososome assembly was monitored using native agarose gel electrophoresis as described (Lamberg *et al.*, 2002). In this gel retardation assay, transpososomes are visualized as protein–DNA complexes that can withstand a challenge by heparin embedded in the gel. Both Em-Mu and Km-Mu produced transpososomes in similar amounts (data not shown), and the yields were comparable to those observed in previous studies with analogous mini-Mu transposons (Lamberg *et al.*, 2002). The two transpososome preparations were then concentrated approximately tenfold by centrifugal cartridges, and the preparations were desalted prior to electroporation (see Methods). Analytical gel retardation assay verified successful concentration of transpososomes and indicated that the pre-assembled complexes tolerated the treatments without disassembly (data not shown).

### Electroporation

Results from the studies with Gram-negative bacteria (Lamberg *et al.*, 2002) indicated that the major factors affecting the colony-formation capacity of each recipient strain include the method of preparing electrocompetent cells and the electroporation parameters. Therefore, we first optimized these factors for each strain studied by using replicative plasmids for electroporation and by varying cell culture conditions and pulse parameters (data not shown). In parallel, viable counts were determined following electroporation to evaluate corresponding cell survival rates. The optimized conditions varied among species and

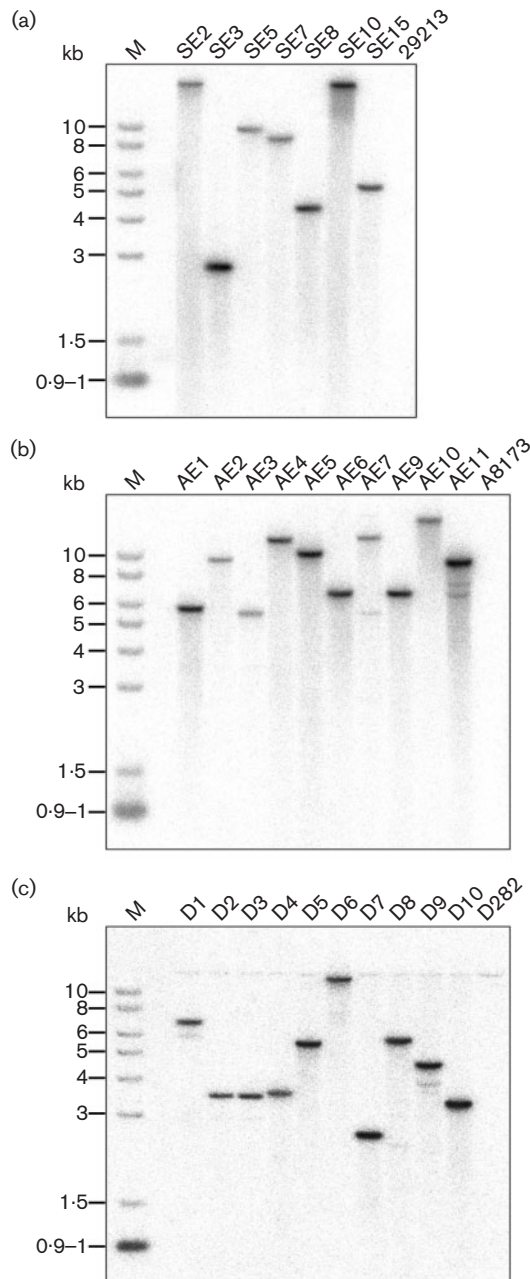
strains (see Methods), and electroporation efficiencies ranged from  $5 \times 10^4$  to  $4 \times 10^6$  c.f.u. ( $\mu\text{g}$  introduced plasmid DNA)<sup>-1</sup> (Table 1). With all strains, approximately 50% of the cells survived electroporation under the optimized conditions. For genomic integration, an aliquot of the concentrated and desalted assembly reaction was electroporated into electrocompetent *Staph. aureus*, *Strep. pyogenes* or *Strep. suis* cells, and bacterial clones were selected for resistance of appropriate antibiotic. The colony-forming capacity among species ranged from  $1 \times 10^1$  to  $2 \times 10^4$  c.f.u. ( $\mu\text{g}$  introduced transposon DNA)<sup>-1</sup>. Notably, these values correlated with the electroporation efficiency values obtained with replicative plasmids, being consistently two to three orders of magnitude lower (Table 1); thus, the results underscored the importance of optimizing the method of preparing competent cells. Only those samples that contained detectable protein–DNA complexes yielded antibiotic-resistant colonies.

### Genomic integration

Southern blot analysis with transposon-specific probes can be used to evaluate the presence and copy number of integrated transposons within genomic DNA. Digestion of chromosomal DNA with an enzyme that does not cleave the transposon DNA generates one fragment that hybridizes to the probe for each integrated transposon copy. Chromosomal DNA from seven *Staph. aureus* Em<sup>R</sup>, ten *Strep. pyogenes* Em<sup>R</sup>, and ten *Strep. suis* Km<sup>R</sup> clones was isolated, digested with *PvuII* (which does not cut the transposon sequence), and analysed by Southern hybridization with appropriate transposon probes. All isolates generated a single, prominent band with a discrete and, for each clone, different gel mobility (Fig. 2). Control DNA from the recipient strains did not produce detectable bands in the analyses. These and additional data obtained by using several other restriction enzymes for chromosomal DNA digestion (data not shown) indicated that a single copy of the transposon DNA was present in the bacterial chromosome of each of the antibiotic-resistant clones studied.

### Chromosomal location of integrated transposons

In general, transposon integration results in the duplication of the target sequence, manifested as a direct repeat that flanks the integrated transposon. In Mu transposition, this target site duplication is 5 bp in length (Allet, 1979; Haapa *et al.*, 1999a; Haapa-Paananen *et al.*, 2002; Kahmann & Kamp, 1979). To investigate the nature of the transposon insertions, we cloned transposon-genomic DNA borders from a total of 31 antibiotic-resistant clones of different strains and determined the sequences on both sides of the transposon by using transposon-specific primers (see Methods). DNA sequence determination revealed a perfect 5 bp target site duplication in each clone (Table 2), thereby confirming that the integrations were generated via authentic transposition and not by other types of DNA-restructuring reactions. The insertion sites within



**Fig. 2.** Southern blot analysis of insertions into the bacterial chromosome. Genomic DNAs of seven Em<sup>R</sup> *Staph. aureus* ATCC 29213 clones (a), 10 Em<sup>R</sup> *Strep. pyogenes* A8173 clones (b), and 10 Km<sup>R</sup> *Strep. suis* D282 clones (c) were digested with *Pvu*II, which does not cut the transposon, and probed with Em-Mu or Km-Mu transposon DNA, as appropriate. Lanes: M, Fermentas MassRuler Mix molecular size markers; SE2–SE15, *Staph. aureus* transposon insertion mutants; 29213, genomic DNA of the original *Staph. aureus* ATCC 29213 recipient strain as a negative control; AE1–AE11, *Strep. pyogenes* transposon insertion mutants; A8173, genomic DNA of the original *Strep. pyogenes* A8173 recipient strain as a negative control; D1–D10, *Strep. suis* transposon insertion mutants; D282, genomic DNA of the original *Strep. suis* D282 recipient strain as a negative control.

chromosomes were then mapped by the aid of heterologous microbial genome sequences available in databases (Table 2). Transposons were distributed in different genomic locations; both ORFs and intergenic regions were represented in the collection of targeted loci.

## DISCUSSION

In principle, a good starting point for the genetic analysis of any bacterial process is an initial generation of a library of insertion mutations. The collection should preferably contain a single insertion within a given genome, the distribution of the mutations should be random, and the number of clones in the library should be high enough to guarantee that each gene within the genome has been mutated at least once. Our present study describes a straightforward and general approach to introduce genetic material into the genomes of Gram-positive bacteria that should fulfil these criteria and allows generation of insertion mutant libraries without evident species barrier(s).

The methodology is based on electroporation of *in vitro*-assembled Mu DNA transposition complexes that become activated for transposition chemistry within bacterial cells and subsequently integrate the delivered exogenous DNA into the genome of the recipient cell, as previously shown with Gram-negative bacteria (Lamberg *et al.*, 2002). However, to be useful with Gram-positive bacteria, the original strategy needed to be modified in two critical ways. First, we utilized mini-Mu transposons containing gene expression cassettes that are known to be functional in Gram-positive bacteria, and which confer resistance to the common antibiotics Em and Km, even when present as a single genomic copy (Autret *et al.*, 2001; Qiao *et al.*, 1996). Second, an important transpososome concentration step was introduced in the protocol, which effectively increased the number of complexes available for penetration into cells in a given electroporation experiment.

Our data demonstrate the usefulness of the Mu transpososome delivery-based genomic DNA integration strategy with three pathogenic Gram-positive species: *Staph. aureus*, *Strep. suis* and *Strep. pyogenes*. The strategy was particularly efficient with *Staph. aureus*, and more than 10<sup>4</sup> antibiotic-resistant colonies were obtained per microgram of input transposon DNA (Table 1). Such a high number guarantees an easy generation of exhaustive mutant libraries for this species. However, as the efficiency was lower in the other two species studied, construction of very large libraries for *Strep. suis* and *Strep. pyogenes* would require scaling up the procedure, e.g. by electroporating more aliquots of competent cells and/or further optimizing the procedures of preparing competent cells for these species. We were not able to use Em-Mu for mutagenesis of *Strep. suis*, as this strain was naturally resistant to Em. In addition, the Km<sup>R</sup> cassette did not confer resistance to Km in *Staph. aureus*, even when present within in a multicopy plasmid, perhaps reflecting differences in the mechanisms of how the

studied bacterial strains recognize critical translation signals. These results underscore the importance of preliminary tests prior to any planned mutagenesis projects, i.e. the strain aimed for mutagenesis should be properly tested with regard to its resistance properties and the functionality of the planned resistance-determining cassette.

Electroporation efficiencies are usually higher in Gram-negative bacteria than in Gram-positive bacteria, due most likely to structural differences between the Gram-positive cell wall and the Gram-negative cell envelope. Electroporation efficiencies with *Strep. pyogenes* and *Strep. suis* were higher when fresh cells were used (data not shown). In contrast, the electroporation efficiency with *Staph. aureus* was highest when frozen cells were used, emphasizing the importance of optimizing for each given strain the conditions and treatments ultimately leading to electrocompetence of the cells. In general, substances that inhibit the synthesis of the peptidoglycan cell wall of Gram-positive bacteria are beneficial when included in the growth medium (Eynard & Teissié, 2000). These reagents most probably directly affect the rigidity of the cell wall and make it more permeable to the incoming DNA. For this purpose, we used glycine in our experiments with *Strep. suis*. However, alternative reagents, such as penicillin G or DL-threonine, may also be used (Mercenier & Chassy, 1988). Optimal electroporation pulse may also vary among different strains. Accordingly, prior to experimenting with transposomes, the optimal pulse parameters should be determined for each strain by using a replicative control plasmid. Notably, the results of such control electroporation experiments are indicative of the usefulness of the Mu transposome strategy; so far the yield of transposon-containing clones has correlated with the observed competence status of a given bacterial strain (Lamberg *et al.*, 2002; this study). In summary, for the generation of sizeable mutant banks, reasonable, but not necessarily very high, electroporation efficiencies are required. Because multiple consecutive electroporations can be performed without too much extra effort, electroporation efficiencies ranging from  $10^4$  to  $10^5$  c.f.u. ( $\mu\text{g}$  replicative plasmid DNA) $^{-1}$  should be high enough for the generation of sizeable mutant libraries, which are adequate, for example, for microbial pathogenesis studies to identify novel virulence genes.

For the generation of mutant banks and experiments thereafter, it is essential that each individual clone contains only one newly introduced mutation. This was the case in the present study, as each analysed clone included a single integrated transposon. In addition, all of these clones contained an accurate 5 bp target site duplication, indicating that the gapped Mu transposition DNA intermediate with single-stranded regions can be repaired correctly by the host machinery of Gram-positive bacteria, and that the possible presence of host-encoded restriction systems or proteases does not impede MuA-mediated transposition. Furthermore, only those samples that contained detectable

protein–DNA complexes yielded antibiotic-resistant colonies, reflecting the inability of the studied cells to efficiently recombine the incoming naked transposon DNA. Our protocol yielded a large number of integrant clones, indicating that the methodology is extremely suitable for the construction of exhaustive mutant libraries. For an average 2.0 Mb microbial genome, when an average ORF is estimated to be 1 kb, a tenfold per-gene coverage would require 20 000 individual mutant clones within a given library. Such a comprehensive mutant library has now been generated for *Staph. aureus*, and its analysis has revealed novel genes involved in bacterial biofilm formation (P. H. Tu Quoc, P. Genevaux, M. I. Pajunen, H. Savilahti, J. Schrenzel and W. L. Kelley, unpublished data).

Similar to Mu transposome mutagenesis of Gram-positive staphylococci and streptococci (this study), Tn5 transposomes have been used to directly mutagenize Gram-positive corynebacteria, mycobacteria and rhodococci (Derbyshire *et al.*, 2000; Fernandes *et al.*, 2001; Laurent *et al.*, 2003; Oram *et al.*, 2002; Takayama *et al.*, 2003; Tanaka *et al.*, 2002). In most cases, the reported genomic integration efficiencies were similar to those obtained in our study, and in some cases they were even higher. However, a direct comparison between the characteristics of these two related systems has not been documented.

Besides efficiency, the spectrum of selected target sites at the sequence level is a critical issue with regard to the usefulness of a given genomic mutagenesis strategy. At least *in vitro*, the Mu system selects its transposition target sites very randomly and without a strict sequence preference (Butterfield *et al.*, 2002; Haapa-Paananen *et al.*, 2002). Essentially identical targeting preference with only a weak selectivity has also been observed in an *in vivo* study that employed the expression of MuA transposase within *E. coli* and integration site analysis from plasmid targets (Mizuuchi & Mizuuchi, 1993). On the basis of the above studies, it is highly probable that a similar pattern will emerge with the MuA-catalysed genomic integration strategy as well, although we acknowledge that the conditions within cells may affect targeting to some extent. Determination of a statistically relevant number of transposon insertion sites within a given bacterial genome will ultimately reveal the targeting preference in each studied strain.

We have extended the Mu-transposome-mediated genomic transposon integration methodology to Gram-positive bacteria, in particular to staphylococci and streptococci, allowing direct generation of sizeable insertion mutant libraries for genomics studies. In principle, it should be possible to use a similar strategy with other Gram-positive bacterial species, given that a reasonable protocol for the preparation of competent cells is available. Furthermore, the mini-Mu transposons can easily accommodate modifications that would enable their advanced usage, e.g. for the generation of reporter gene fusions (Hayes, 2003) and for signature-tagged mutagenesis (Hensel *et al.*, 1995).



## ACKNOWLEDGEMENTS

We thank Pirjo Rahkola and Sari Tynkkynen for excellent technical assistance. Plasmid pLEB620 was constructed in collaboration with Justus Reunanen and Per Saris (University of Helsinki, Finland). Financial support was obtained from the Academy of Finland (separate grants to M.I.P., J.F. and H.S.), and from the Finnish National Technology Agency TEKES (to H.S.).

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