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New Vector for Efficient Allelic Replacement in Naturally Nontransformable, Low-GC-Content, Gram-Positive Bacteria†

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A shuttle vector designated pMAD was constructed for quickly generating gene inactivation mutants in naturally nontransformable gram-positive bacteria. This vector allows, on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) plates, a quick colorimetric blue-white discrimination of bacteria which have lost the plasmid, greatly facilitating clone identification during mutagenesis. The plasmid was used in *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* to efficiently construct mutants with or without an associated antibiotic resistance gene.

The study of chromosomal genes and the modification of bacterial strains require the development of new strategies and new genetic tools to facilitate the rapid screening of recombinant clones. Molecular genetic analyses require the exchange by homologous recombination of a chromosomal gene by a mutated allele or inactivated copy, and several strategies have been developed for generating gene replacements in bacterial chromosomes. Here, we describe a new plasmid for efficiently accomplishing such constructions in nontransformable bacteria. This plasmid, pMAD, overcomes the limitations of the available vectors, which were largely developed for highly transformable bacteria. For example, a one-step gene inactivation procedure has been developed for *Bacillus subtilis* by using a nonreplicative vector called pMutin (22). This vector carries a *lacZ* reporter gene and an inducible Pspac promoter which can be induced by IPTG (isopropyl- β -D-thiogalactopyranoside). Following chromosomal integration via a single crossover event of a pMutin recombinant vector containing an internal fragment of the target gene, the target gene is inactivated, *lacZ* becomes transcriptionally fused to the gene, and the Pspac promoter controls the transcription of the downstream gene(s) in an IPTG-dependent fashion.

To avoid the introduction of a complete plasmid and duplication of the target sequence in the chromosome, other strategies were described. These two-step strategies proceed by homologous recombination between a target gene and homologous sequences carried on a plasmid which is temperature sensitive for DNA replication. After transformation of the plasmid into the host, integration of the plasmid into the chromosome by a single crossover event is selected during growth at the nonpermissive temperature while maintaining selective pressure. Subsequent growth of the cointegrates at the permis-

sive temperature (30°C) leads to a second recombination event, resulting in their resolution. Screening of clones where an efficient second recombination event has taken place (i.e., where the loss of the vector is concomitant with the deletion of the gene of interest) is rather difficult without positive selection or colorimetric screening of the candidate clones. A general system for generating gene replacement in bacterial chromosomes was developed by using the pORI vector (12). This conditionally replicating vector contains the *Escherichia coli lacZ* gene, which is expressed from a constitutive promoter recognized in gram-positive and gram-negative bacteria. The presence or the absence of the *lacZ* gene enables a simple blue-white screening of recombination events. However, these plasmids lack the *repA* gene encoding the replication initiation protein and are unable to replicate in any bacterial strains unless the RepA protein is provided in *trans*. In consequence, high transformation frequencies are required to obtain integrants and deletions or substitutions are produced after resolution of cointegrants, thus limiting the use of this plasmid vector to naturally competent bacteria with a high efficiency of transformation (12). A system for generating deletions in open reading frames of *E. coli* was developed by using plasmid pK03, which contains a temperature-sensitive origin of replication, a chloramphenicol resistance gene, and a counterselectable *sacB* gene to facilitate allelic exchange. Briefly, the *in vitro* altered sequences carried on the vector pK03 are integrated into the chromosome by using the sucrose-resistant phenotype, and the sucrose-resistant and chloramphenicol-sensitive colonies are screened for the desired gene replacement (15). In earlier work with the Tn917 transposon in *B. subtilis* (25), methods of recovering populations of bacteria containing chromosomal insertions of the transposon were based on the use of temperature-sensitive vectors derived from a small *Staphylococcus aureus* plasmid called pE194 (9). However, practical complications arose from the fact that the thermosensitivity of replication of the pE194-derived vectors was incomplete. Replication of the plasmid was impaired at high temperatures but not abolished. New vectors based on the use of pE194^{ts}, a thermosensitive mutant derivative of pE194, were isolated by Alexan-

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† This paper is dedicated to the memory of Maryvonne Arnaud, who died on 1 February 2004.

dra Gruss in R. Novick's laboratory (A. Gruss, personal communication) (24). These vectors display an extremely tight replication block above 37°C yet maintain essentially wild-type copy numbers at temperatures below 32°C. A chimeric integrational vector called pKSV7, which contains temperature-sensitive replication functions derived from the pE194^{ts} that functions in *B. subtilis*, was constructed (20). As shown by Ehrlich and Noirot (17), the recombination excision of integrated plasmids is greatly stimulated in the vicinity of an active replication origin. It was shown that excision rates of integrated pKSV7 were greater than with ordinary ColE1-derived vectors, facilitating the introduction of deletions into chromosomal genes (20). The thermosensitive replication origin of pE194^{ts} was also used to construct delivery vectors for obtaining chromosomal insertions of Tn917 in *B. subtilis* (19) and in *Listeria monocytogenes* (2).

In contrast to the situation observed with *Streptococcus pneumoniae* and *B. subtilis*, where the natural development of competence is concomitant with the increased expression of recombination genes, allelic replacement is a time-consuming and fastidious task in nonnaturally transformable bacteria, such as *S. aureus*, *Bacillus thuringiensis*, or *L. monocytogenes*. A shuttle vector (pE194^{ts}::pBR322), which replicates in both *E. coli* and *S. aureus*, was constructed (A. Gruss, unpublished results). This vector has a thermosensitive pE194 replication origin (24) and confers erythromycin resistance to gram-positive hosts. This vector was used successfully for gene inactivation in *B. thuringiensis* (13, 14). However, the use of this vector or similar constructs, such as pKSV7, to obtain mutants remained labor intensive, due to the low efficiency of recombination and the lack of a simple method for screening recombinant clones where excision of the plasmid has taken place. In the postgenome era, there is an obvious need to develop vectors that facilitate the high-throughput mutagenesis required to analyze novel regulatory genes and potential virulence determinants in nonnaturally transformable gram-positive pathogens, such as *S. aureus* and *L. monocytogenes*. To this end, we have constructed a new vector called pMAD combining the different advantages of the various plasmids described above. This vector carries a constitutively expressed transcriptional fusion with the *bgab* gene encoding a thermostable β -galactosidase from *Bacillus stearothermophilus*, allowing the easy screening of transformants on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) plates.

Construction of a novel vector, pMAD, for efficient gene replacement in nonnaturally transformable gram-positive bacteria. The pMAD vector was constructed by using a replication-thermosensitive mutant of pE194. This plasmid, conferring resistance to the macrolide, lincosamide, and streptogramin B antibiotics, was originally isolated from *S. aureus* and transferred to *B. subtilis* (7, 24). Plasmid pE194^{ts} was linearized at the single ClaI restriction site and cloned into the corresponding site of pBR322 (1) to generate the shuttle vector called pE194^{ts}::pBR322 (A. Gruss, personal communication).

In the first step, the EcoRI restriction site of pE194^{ts}::pBR322 plasmid was filled in with the Klenow fragment of DNA polymerase I in the presence of the four deoxynucleoside triphosphates and religated with T4 DNA ligase. Three DNA fragments were cloned between the NruI and the EcoRV restriction sites of the shuttle vector (see Fig. 1A): (i) a NaeI-

BglII DNA fragment containing the extended multiple cloning site from the pMTL22 plasmid (3); (ii) a chromosomal BglII-BamHI DNA fragment (128 bp) containing the constitutively expressed promoter region of the *S. aureus clpB* gene encoding a Clp-Hsp100 ATPase (5, 6) and amplified by PCR from chromosomal DNA of strain RN6390 of *S. aureus* (18) with the Pwo thermostable DNA polymerase and oligonucleotides O₁ (5'-G GAAGATCTGTCTAGTTAATGTGTGAACGTA-3') and O₂ (5'-CGCGGATCCGACCTTAATTATATTATAGTCCCAA T-3'); and (iii) a BamHI-StuI DNA fragment containing the promoterless *bgab* gene from *B. stearothermophilus* encoding a thermostable β -galactosidase (8). A map of the resulting plasmid, pMAD, is shown in Fig. 1B. Expression of *bgab* in pMAD is driven by the constitutive *pclpB* promoter.

Utilization of the pMAD vector for gene replacement in *S. aureus*. To assess the pMAD vector for the generation of stable chromosomal mutations in *S. aureus*, we constructed a derivative of pMAD carrying the upstream part of a target gene, an antibiotic resistance cassette (spectinomycin resistance), and the downstream part of the target gene. We chose to inactivate two contiguous genes called *vraF* and *vraG* (*vra* standing for vancomycin resistance associated). These genes are up-regulated in *S. aureus* strains Mu3 and Mu50. The encoded proteins constitute a likely ABC transporter. This ABC transporter gene was found to be transcribed in a greater amount in the Mu50 strain, and *vraFG* probably contributes to vancomycin resistance through the transport of the cell wall amino acid component (11). DNA fragments corresponding to the upstream region of *vraF* (extending from positions -665 to -33 with respect to the *vraF* translation initiation site) and the downstream region of *vraG* (extending from positions -57 to +805 with respect to the *vraG* stop codon) were amplified with the oligonucleotide pairs OMD189 and OMD190 (5'-GGAG GATCCTCGATGCTGGATACACAGCTG-3' and 5'-CTCC TCGAGCCTTTAGGCTTTGGCACTTG-3', respectively) and OMD191 and OMD192 (5'-AGAAGATCTGCTGTTTTTGC AGTGACGGC-3' and 5'-GGAGGATCCCGAATTCAATA GCAGCACG-3', respectively), the high-fidelity Triple Master polymerase (Eppendorf), and chromosomal DNA from strain RN6390 as a template. The PCR products were treated with XhoI and BglII restriction enzymes, respectively. A 1.3-kb DNA fragment containing the entire spectinomycin resistance gene *spc* (16) was amplified from plasmid pIC333 (21) with the oligonucleotides OMD156 (5'-AGAAGATCTCACCTAGAT CCTTTTACTC-3') and OMD157 (5'-CTCCTCGAGAAA GTAAGCCTGTTATTGC-3') and was restricted with the XhoI and BglII restriction enzymes.

These purified PCR DNA fragments were mixed in equal amounts and ligated with DNA ligase. The ligation mixture was used as a template for the Triple Master polymerase to produce the complete tripartite DNA fragment by PCR with OMD189 and OMD192 oligonucleotides. The full-length 2.4-kb PCR fragment was then purified on an agarose electrophoresis gel, restricted with BamHI, and cloned in the corresponding restriction site of the pMAD vector. The recombinant pMAD Δ FG vector was extracted from *E. coli*, and a two-step procedure was then used for allele replacement in *S. aureus*, as detailed below.

In the first step, the recombinant pMAD Δ FG vector was introduced by electroporation into *S. aureus* strain RN4220

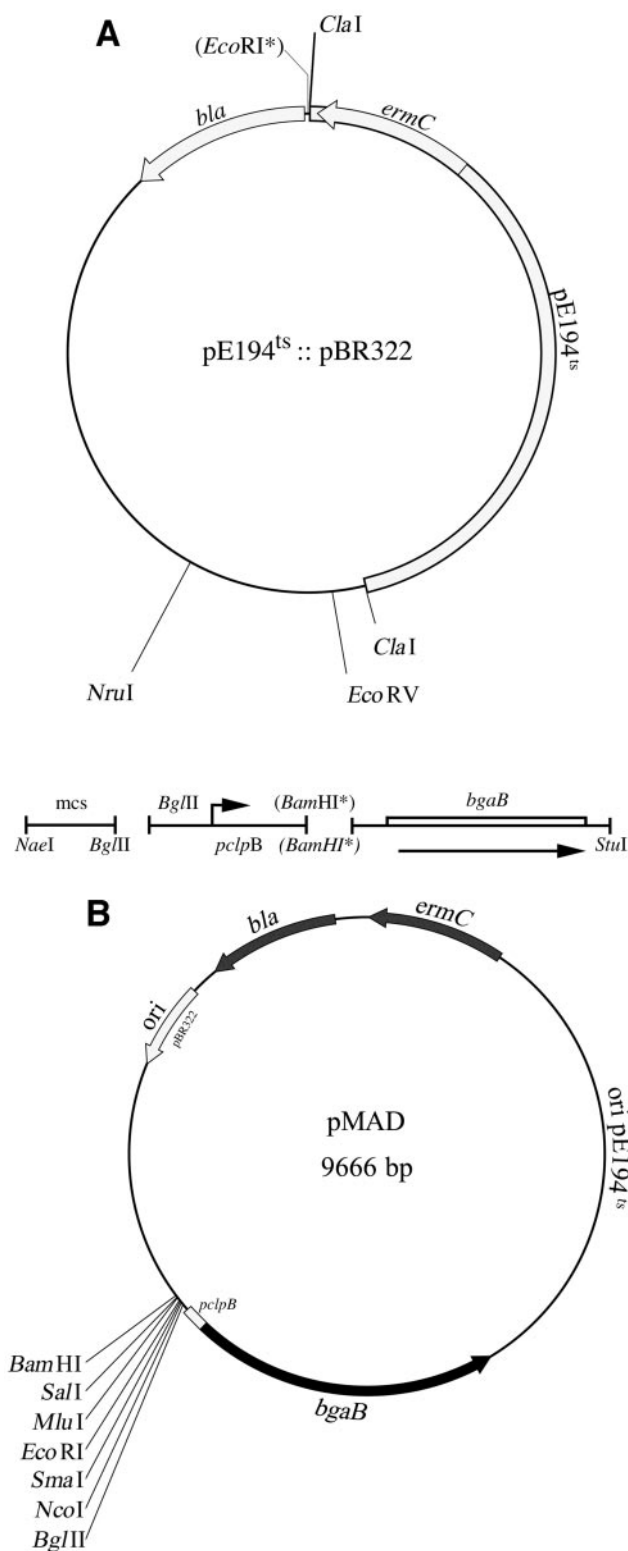


FIG. 1. Physical map of pE194^{ts}::pBR322 and its derivative pMAD. (A) Construction of the pMAD plasmid. DNA fragments used to construct pMAD are indicated: mcs, multiple cloning site; *pclpB*, DNA fragment from *S. aureus* containing the *clpB* promoter region; *bgaB*, DNA fragment containing the promoterless *bgaB* gene encoding a thermostable β -galactosidase (8). Asterisks indicate restriction sites which have been lost during the construction steps. (B) Map of the pMAD plasmid. Unique restriction sites are indicated.

(10). Transformants (10^6 transformants/ μ g of DNA) were selected after 2 days at 30°C on rich medium (tryptic soy broth; Difco) containing either spectinomycin (100 μ g/ml) or erythromycin (5 μ g/ml).

One blue colony was streaked onto the same medium and incubated at 30°C. In the second step, a single blue colony was inoculated in tryptic soy broth complete medium without antibiotic at an optical density at 600 nm of 0.01 and incubated with shaking for 2 h at 30°C followed by 6 h at 42°C. Serial dilutions of this culture were plated on tryptic soy agar (TSA) containing X-Gal and spectinomycin and incubated at 42°C overnight. Among a vast majority of light blue clones due to the integration of the vector by a single crossover event, 1 to 5% of white colonies were present and represent candidate clones resulting from a double crossover event and loss of the vector (Fig. 2). Several white colonies were isolated on the same medium at 37°C and verified for erythromycin sensitivity. To confirm the gene deletion, chromosomal DNA was extracted from five candidate clones and used in PCRs with oligonucleotides OMD202 (5'-GCGGAAACGCCATTTCAA CG-3') and OMD203 (5'-GCTGCCGTTTCTTCAATTGC-3'), hybridizing 926 bp upstream from the *vraF* translational start site and 1,006 bp downstream from the *vraG* stop codon, respectively. All of the spectinomycin-resistant, erythromycin-sensitive white colonies on X-Gal-containing plates had the expected deletion and replacement of the *vraF/vraG* locus. The pMAD vector was also used to create various deletions in the *sarA* and *sigB* genes in clinical isolates of *S. aureus* strain 15 981, producing biofilms. In addition to the well-characterized roles of SarA in the regulation of many virulence determinants, it has been shown that SarA plays a role in the regulation of biofilm development (23). The pMAD vector also replicates at 30°C in other gram-positive bacteria and was successfully used to create gene replacements in *L. monocytogenes* and *Bacillus cereus* as we describe here.

Gene replacement in *L. monocytogenes*. A similar strategy was used to inactivate the *ctsR* gene in *L. monocytogenes* strain LO28 (4). In this mutant, the *ctsR* gene was replaced through a double crossover event by the *aphA3* kanamycin resistance gene deprived of its transcription initiation and termination sequences. However, the introduction of an antibiotic resistance marker can sometimes be undesirable for various reasons, such as the polar effect of the inserted DNA on the expression of the downstream genes in an operon or the multiple introductions of DNA fragments or different mutations within the same strain. To construct a deletion without insertion of foreign DNA, the *clpB* gene of *L. monocytogenes* was chosen as a target. A deletion eliminating the entire coding sequence of the *clpB* gene was constructed by generating through PCR two DNA fragments, of 829 and 761 bp, with oligonucleotides AC189 and AC190 (5'-AATGGATCCCACA TCGGAGCGAGTAAACAC-3' and 5'-TAAGTCGACTCAT TCGTCCTCCTTATAAAA-3', respectively) and AC192 and AC193 (5'-CACGTCGACTGAAAGGGAAAAC TTG GTTG-3' and 5'-TATCCATGGAATATTTATTTACTGGTT TTA-3', respectively), corresponding to the chromosomal DNA regions located directly upstream and downstream, respectively, from the *clpB* gene. These two DNA fragments were cloned in tandem in pMAD, resulting in plasmid pMAD Δ *clpB*. A three-step procedure was used to obtain the

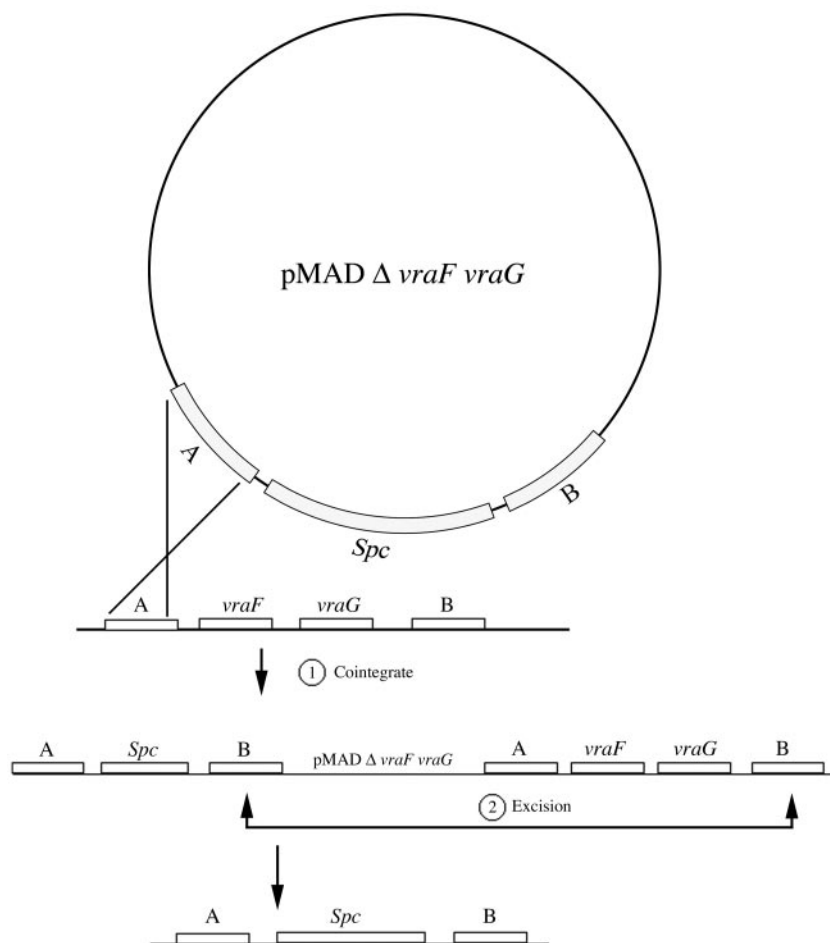


FIG. 2. Schematic representation of a two-step procedure used to obtain gene replacement recombination. Areas labeled A and B represent DNA sequences located upstream and downstream from *vraF* and *vraG* genes. The crossed lines indicate crossover events. The integration of pMAD via homologous sequences can take place in area A or B. The cointegrate undergoes a second recombination event, regenerating the pMAD plasmid. Depending on whether the second recombination event occurs between the two homologous sequences in area A or B, the spectinomycin resistance marker will either remain in the chromosome (area B) or be excised along with the plasmid (area A). Gene replacement occurs only if the second recombination event occurs in area B, as shown.

L. monocytogenes mutant. The pMAD Δ *clpB* plasmid was introduced by electroporation into strain LO28 of *L. monocytogenes*, and transformants were selected at 30°C on brain heart infusion (BHI) plates containing erythromycin and X-Gal (50 μ g/ml). One blue clone was reisolated on the same medium and incubated in BHI liquid medium at 39°C. To obtain integrants resulting from a single crossover event via homologous sequences, serial dilutions were plated on BHI erythromycin (5 μ g/ml) plates containing X-Gal (50 μ g/ml) and incubated at 39°C for 2 days. Clones resulting from the integration of the pMAD Δ *clpB* plasmid into the *clpB* gene were blue. Several blue colonies were cultivated at 30°C for 6 h, followed by incubation at 39°C for 3 h; dilutions were plated on TSA X-Gal plates in the absence of antibiotics. The appearance of white colonies (10 to 30%) on X-Gal plates indicates the excision and loss of the plasmid vector (Fig. 3). Five colonies were checked for erythromycin sensitivity, and PCR amplifications with oligonucleotides located upstream and downstream from the *clpB* gene were performed to confirm the gene deletion.

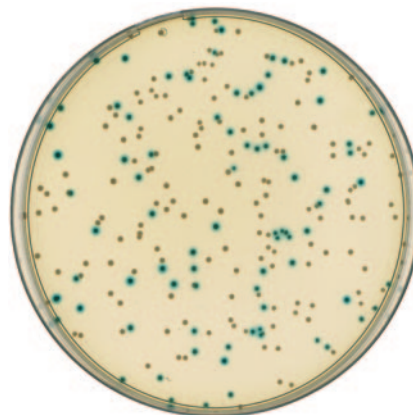


FIG. 3. *L. monocytogenes* LO28 colonies following transformation with the pMAD Δ *clpB* plasmid. Cells were cultivated at 30°C for 6 h, followed by incubation at 39°C for 3 h; dilutions were plated on TSA X-Gal plates in the absence of antibiotics. White colonies have undergone the excision and loss of the plasmid vector, whereas blue colonies retain a copy of the plasmid integrated in the chromosome.

One clone was shown to contain the expected deletion in *clpB* after the resolution process (4).

Gene replacement in *B. cereus*. The pE194^{ts}::pBR322 plasmid has been used previously to construct mutants of *B. thuringiensis* (13, 14). However, the low frequency of transformation and recombination and the lack of a clear phenotype allowing the screening of colonies where successful crossover events have taken place render the use of this plasmid tedious in this host. The pMAD vector was used successfully to delete the *tetB* gene (open reading frame RZC00709; Integrated Genomics, Inc.) from *B. cereus* strain ATCC 14579. Two DNA fragments of 877 bp and 1,013 bp corresponding to the regions located directly upstream and downstream from *tetB* were amplified by PCR with the following pairs of oligonucleotides: Tet1 and Tet2 (5'-CATGCCATGGCAGTAAGACCTCCAT CTC-3' and 5'-CCGGAATTGCGAAACACTTCATCTGTG TG-3', respectively) and Tet3 and Tet4 (5'-CCCAAGCTTCG TCATTTAATTGCTGCTC-3' and 5'-CGCGGATCCTGTGTTAAGAAGAGATGATCGC-3', respectively).

These two DNA fragments were cloned in the pMAD vector on either side of a DNA fragment containing the *spc* spectinomycin resistance gene, producing the pMAD Δ *tetB* plasmid. This plasmid was introduced by electroporation into *Bacillus cereus*, and transformants were obtained after 2 days at 30°C on Luria-Bertani (LB) plates containing 250 μ g of spectinomycin/ml, 3 μ g of erythromycin/ml, and 50 μ g of X-Gal/ml. A pool of individual blue clones was used to inoculate a culture of LB medium without antibiotic incubated at 40°C to stationary phase. Two more cycles of growth were carried out by diluting the stationary phase culture into fresh medium. Finally, single colonies were isolated by plating dilutions of the overnight culture onto LB plates containing X-Gal and 300 μ g of spectinomycin/ml. Several white colonies were isolated and checked for the erythromycin-sensitive phenotype. Chromosomal DNA was purified from several clones and used to make PCRs with oligonucleotides hybridizing outside the cloned DNA fragments to verify the extent of the deletion and insertion in the *tetB* gene. All the candidate clones were shown to contain the deletion and insertion in the *tetB* gene (S. Fedhila, unpublished results).

We note that although this vector has proven to be a valuable tool and been used successfully in several gram-positive bacteria (*S. aureus*, *L. monocytogenes*, and *B. cereus*), the pE194 origin of replication is not functional in streptococci, enterococci, and lactococci, preventing the use of pMAD in these bacteria (P. Trieu-Cuot, personal communication).

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