Markerless genome editing in competent streptococci

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Abstract

Selective markers employed in classical mutagenesis methods using natural genetic transformation can affect gene expression, risk phenotypic effects, and accumulate as unwanted genes during successive mutagenesis cycles. In this chapter, we present a protocol for markerless genome editing in *Streptococcus mutans* and *Streptococcus pneumoniae* achieved with an efficient method for natural transformation. High yields of transformants are obtained by combining the unimodal state of competence developed after treatment of *S. mutans* with *sigX*-inducing peptide pheromone (XIP) in a chemically defined medium (CDM) or of *S. pneumoniae* with the competence-stimulating peptide (CSP) together with use of a donor amplicon carrying extensive flanking homology. This combination ensures efficient and precise integration of a new allele by the recombination machinery present in competent cells.

Key-words: pheromone, competence, natural transformation, markerless mutagenesis, *Streptococcus mutans*, *Streptococcus pneumoniae*, streptococcus, XIP, CSP

1. Introduction

Genome editing is a powerful tool for the analysis of gene function and regulatory pathways in many organisms. In the bacteria, natural genetic transformation can provide a direct route between synthetic DNA constructs and the cell genome, via DNA uptake and homologous recombination. As the efficiency of this process is often low, many routine strategies for mutagenesis by this route employ a selective marker linked to the desired mutation, allowing recovery of rare recombinants simply by use of selective agar medium. While invaluable for mutagenesis in organisms with low transformation efficiency, such markers unfortunately carry unwanted information, which can potentially alter the organisms' gene expression and phenotype. In addition, during successive cycles of mutagenesis different markers accumulate, compounding their side effects.

To create a simple method of markerless genome editing in *S. mutans* UA159 or in laboratory strains of *S. pneumoniae* , it was necessary to raise the efficiency of transformation close to unity. In *S. mutans*, the recently described competence pheromone known as the *sigX*-inducing peptide (XIP) [1, 2] has the potential of stimulating development of competence in all cells in a population, in contrast to the bimodal response to another *S. mutans* competence pheromone, CSP [3, 4, 5, 6]. In addition, the XIP-induced competent state is unusually persistent in *S. mutans* , lasting for hours and accompanied by a reduced apparent growth rate [7]. In *S. pneumoniae* , high rates of transformation are obtained by treatment with pneumococcal CSP [8] and the use of large DNA fragments. As previously shown, transformation efficiency increases dramatically for genomic donor fragments larger than 1 kb, approaching a maximum only above 10 kb [9, 10].

By combining the aforementioned key factors with use of PCR amplicon donors targeted to a single genomic site, an significant increase in transformation efficiency from 0.1 to 1 % to rates higher than 30 % was obtained in both *S. mutans* and in *S. pneumoniae* [11, 12].

Recovery of the desired mutant can be accomplished by use of a simple PCR step using specific primers that distinguish the mutant from the parental alleles. Given that efficiencies above 30 % are routinely obtained, the screening of one or two dozen colonies is normally sufficient.

This chapter describes a protocol that incorporates these key factors to achieve markerless genome editing in a *S. mutans* reference strain or in *S. pneumoniae* .

2 Materials

2.1 Competence Induction and Transformation

1. *S. mutans* UA159 (ATCC 700610) is a type strain. The *S. pneumoniae* strains are derivatives of the lab strain Rx-1 [9].

2. Agar plates: Tryptic Soy Broth (TSB) 30 g/L (Becton Dickinson, Franklin Lakes, NJ, USA). Add 15 g/L of agar to the medium (VWR Chemicals, Radnor, PA, USA) and autoclave at 121 °C for 15 min. For selective plates, the appropriate antibiotic should be added to the medium once it has cooled below 60 °C and the plates should be stored under appropriate conditions for the antibiotics (*see* **Note 1**).

3. Liquid media: Chemically defined medium (CDM) [6], prepared from stable concentrated stock solutions with 1 % glucose is used for growth, stock preparation, and transformation assays (*see* **Note 2**). Tryptic Soy Broth (TSB, Becton Dickinson, Franklin Lakes, NJ, USA) is used for growth, transformation assays, preparation of stocks, and serial dilutions.

4. Synthetic peptides: Synthetic pheromones can be ordered from custom peptide synthesis services. Lyophilized synthetic XIP (GLDWWSL) is reconstituted with 20 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MI, USA) to which 1 mL of distilled water is

added to give a final concentration of 10 mM (stored at −20 °C). Working stocks are prepared at 100 μM by dilution in distilled water (*see* **Note 3**). Synthetic CSP1 (EMRLSKFFRDFILQRKK) or CSP2 (EMRISRIILDFLFLRKK) for *S. pneumoniae* are dissolved in water at 250 μg/mL.

5. Donor DNA is prepared as PCR amplicons with homologous flanking regions ≥2–3 kb each. Positive control donors can be prepared by PCR amplification of a selective marker flanked by ≥2–3 kb on both sides.

2.2 PCR

1. DNA polymerase: Q5 High-Fidelity DNA polymerase kit (New England Biolabs, Ipswich, MA, USA) is used for construction of amplicons (*see* **Note 4**). For colony screening, TrueStart Hot Start Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) is used.

2. PCR: Any conventional PCR cycler can be used. In the examples shown, the Eppendorf Mastercycler Gradient PCR Thermal Cycler (Eppendorf, Hamburg, Germany) was used.

2.3 Agarose Gel Electrophoresis

1. Agarose: SeaKem LE Agarose (Lonza, Basel, Switzerland) is used to prepare gel for electrophoresis. The concentration of agarose is adjusted according to expected fragment size following the recommendations of the manufacturer.

2. TAE buffer: Tris-acetate-EDTA (TAE) buffer containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA is used to prepare the gel and for running the electrophoresis.

3. DNA stain: SYBR safe DNA gel stain in 10,000× DMSO (Thermo Fisher Scientific, Waltham, MA, USA) is incorporated into the gel at a 1:10,000 proportion with TAE buffer.

2.4 Primers

Oligonucleotide primers are obtained (deprotected and desalted) from commercial synthesis services. Primers are dissolved in molecular biology-grade (sterile deionized) water to obtain a stock concentration of 100 μM, and stored at −20 °C.

3 Methods

The following protocol was established with the objective of achieving sufficiently high yields of transformants to allow convenient direct editing of the genome without resort to selective markers. The nature of each sequence alteration hinges on the research objective and can vary from single base substitutions to gene deletions, rearrangements, or replacements (*see* Subheading 3.4). In *S. mutans* , the basis for the method is the extended maintenance of a high level of competence in CDM, which allows a steady accumulation of recombinants during prolonged exposure to a high-MW donor DNA (*see* Fig. 1).

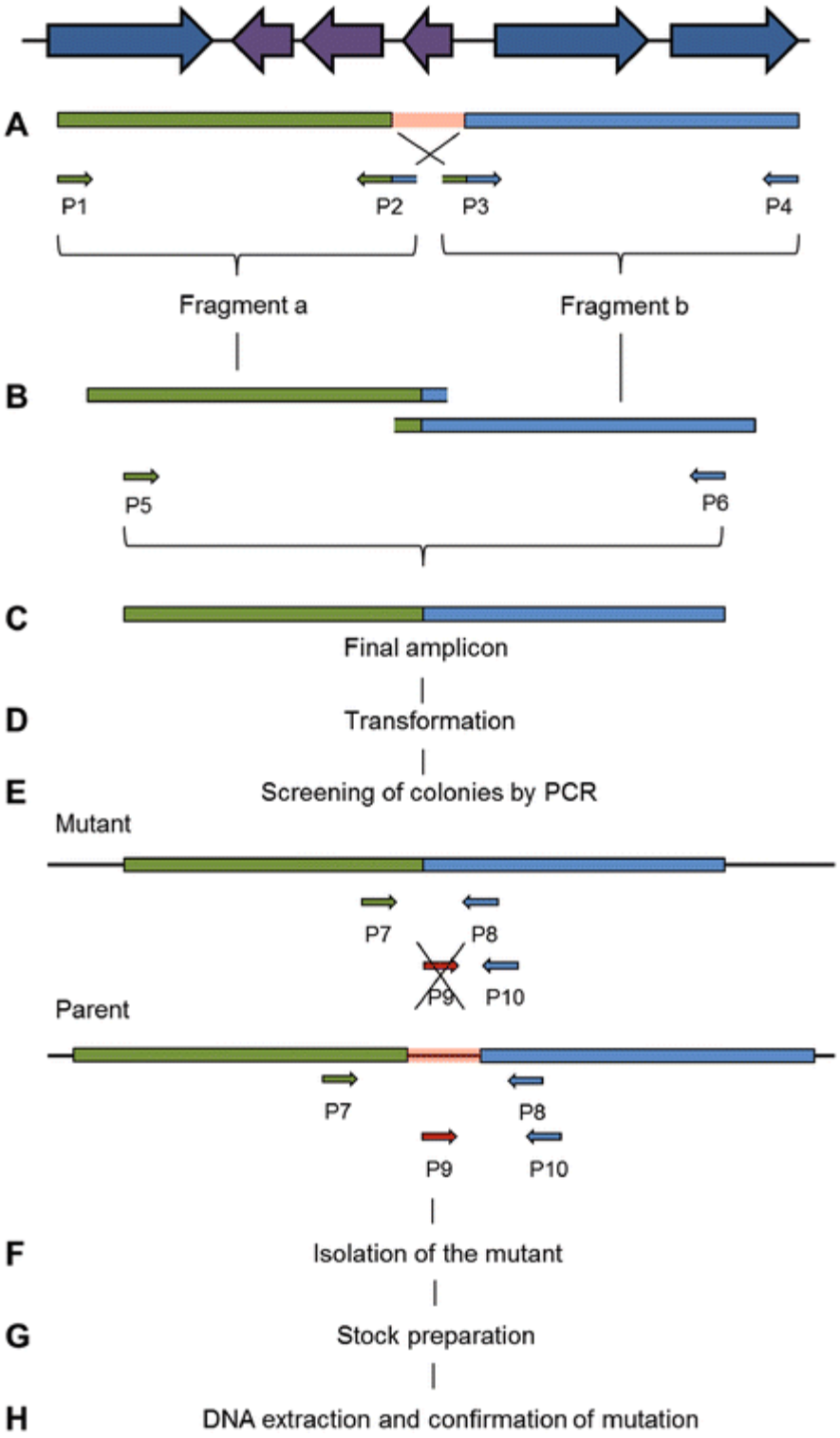

Fig. 1 Comparison of Kinetics of *sigX* expression in CDM and TSB. Strain SM068 was grown in 200-µL volumes of (**a**) CDM or (**b**) TSB in a 96-well plate in ambient air at 37 °C with or without 1 µM XIP (*black*) or 250 nM CSP-18 (*grey*), respectively. The *sigX* expression (*sigX* reporter luminescence measured as relative light units, RLU) relative to the optical density at 600 nm (OD_{600}) of the culture ((**c**) for CDM, (**d**) for TSB) was monitored periodically. The results shown are the averages of three replicates (±SEM)

3.1 Construction of Markerless Amplicons

1. Design primers to amplify two sections in the locus where the mutation will be inserted (Fig. 2a). For illustrative applications *see* Subheading 3.4. In the case illustrated in Fig. 2, the region marked in red will be deleted. Primers P1 and P4 are 18–22 bp in length and have a melting temperature close to 60 °C. Base composition should be 40–60 % G + C and, if possible, the 3′-terminal base should be a G or a C. Primers should be designed to avoid primer dimers or hairpin structures. Primers P2 and P3 overlap and their design is restricted to the specific sequence in the region where the mutation will be inserted. Firstly, design the primer to match a region flanking the desired mutation. Secondly, add to this sequence a similar-sized sequence present in the antisense strand, to overlap with the other amplicon (Fig. 2a). Independently, both of these sequences that will form one primer should have 16–18 bp and a melting temperature close to 60 °C. The final sequences of P2 and P3 will have 34–36 bp each. It is crucial that the flanking regions of homology in each of the amplicons extend for 2–3 kb.

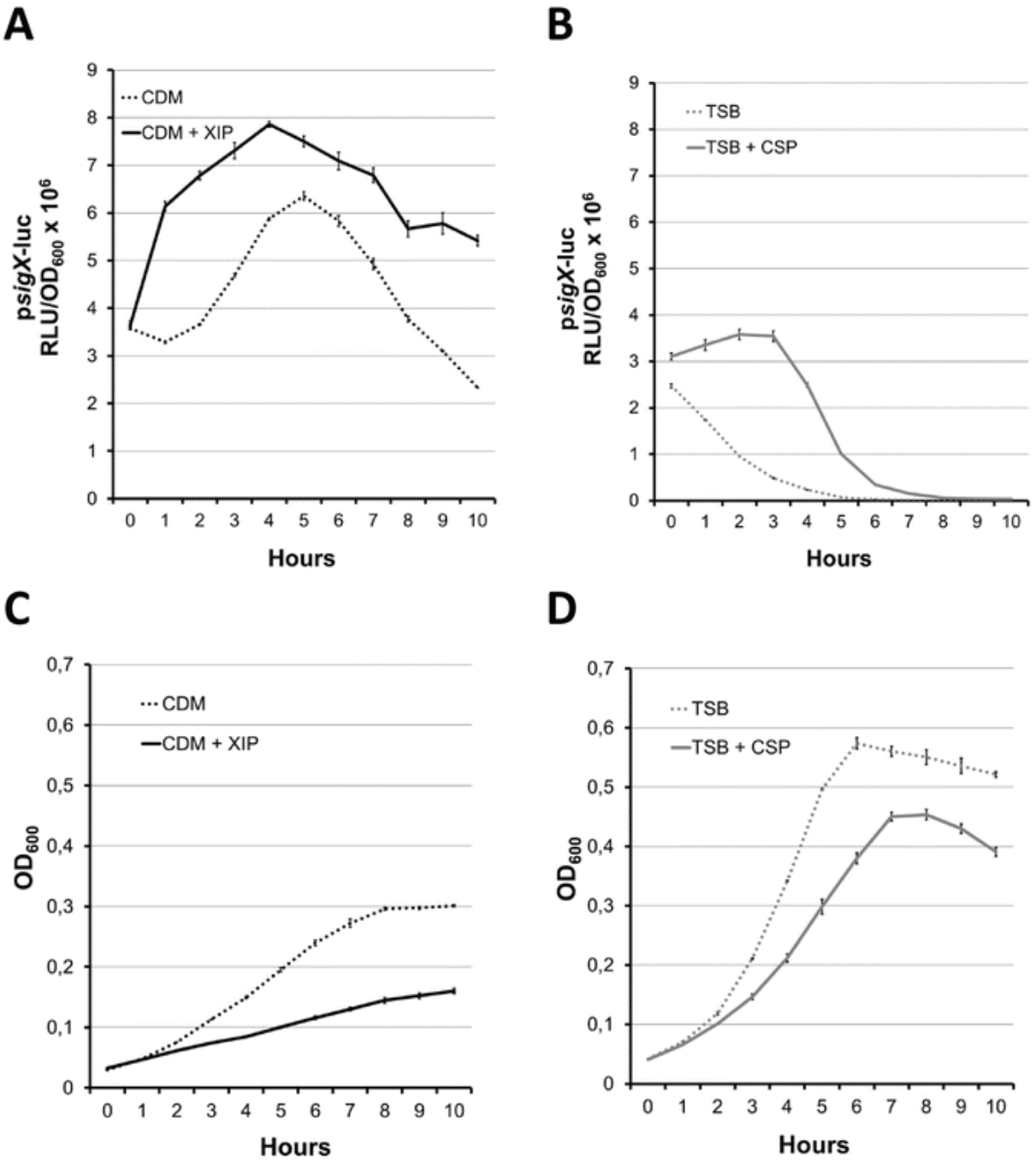

Fig. 2 Workflow for markerless genome editing. Amplicon design and construction (a–c) are prepared according to the desired mutation, exemplified in this diagram as a gene deletion (marked in red). This step is followed by a highly efficient (>35 %) method for natural transformation (d). Two dozen colonies recovered from the transformation step are screened with specific primers that amplify fragments with different sizes in the mutant when

compared to the parent (e). The mutant is then isolated (f, g) and the mutation is confirmed with DNA sequencing (h)

2. Amplify each fragment (a + b) with a proofreading DNA polymerase enzyme that is optimized for accurate amplification of long fragments (*see* **Note 4**). Confirm the presence of the correct fragment by gel electrophoresis.

3. Design two primers that will be used to connect and amplify the fragments (P5 and P6) (Fig. 2b). Using nested primers (P5 and P6), in contrast to using the original outer primers (P1 and P4), greatly increases the specificity of the amplification [13]. As before, utilization of a proofreading DNA polymerase enzyme that is optimized for long fragments is essential (*see* **Note 5**).

4. Confirm the amplification of the correct fragment by gel electrophoresis. Fragments a and b (Fig. 2a) are occasionally observed but weaker than the band for the final amplicon.

5. Utilize the final amplicon as donor DNA to transform the recipient strain (Fig. 2c).

1.2 **Markerless Transformation Protocol Using XIP in *S. mutans***

1. Stock cultures prepared in CDM (OD_{600} 0.5) from fresh colonies are stored with 15 % glycerol at −80 °C (*see* **Note 6**).

2. Dilute the frozen stock 1:10 in fresh CDM; the initial OD_{600} = ~0.05 (see **Note 7**).

3. Incubate at 37 °C until OD_{600} = 0.1 (approximately 2 h).

4. Add XIP to final concentration of 1 μM.

5. Add 50–100 ng of the donor amplicon per mL of culture and incubate at 37 °C for 3 h in a closed 1.5-mL Eppendorf tube (*see* **Note 8**).

6. Prepare serial dilutions of the competent culture in TSB; spread 100 μL of the following dilutions on TSB agar plates—10^{-4}, 10^{-5}, and 10^{-6}.

7. Incubate the plates for 24 h at 37 °C with 5 % CO_2 (*see* **Note 9**).

8. Pick 24 isolated colonies using sterile inoculating loop or pipettor tips; resuspend each in 10 μL of sterile distilled water. Prepare a streak plate from 2 μL of each suspension on a new TSB or THB agar plate (*see* **Note 10**).

9. Prepare a PCR reaction with the following components to test each colony for the intended genome modification (*see* **Note 11**). During screening, P7 and P8 (Fig. 2e) should amplify bands with different sizes in the mutant when compared to the parent, allowing for differentiation (*see* **Note 12**).

Template (from suspended colony)	0.2 μL
Forward primer	0.06 μL

Reverse primer	0.06 μL
10× Reaction buffer	1 μL
10 mM dNTP	0.2 μL
Nuclease-free water	7.28 μL
25 mM $MgCl_2$	1 μL
Hot start *Taq* DNA polymerase	0.2 μL
Total	10 L

10. Thermal cycling parameters:

Description	Temperature	Time
Initial denaturation	95 °C	3 min
25 cycles	95 °C	30 s
	55 °C	30 s
	72 °C	50 s

Description	Temperature	Time
Final extension	72 °C	5 min

11. Analyze the products of each PCR reaction by gel electrophoresis. The results will be either negative (showing only the band for the parent allele), pure positive (showing only the band for the mutant allele), or mixed (showing both bands) (*see* Fig. 3). If a desired transformant is identified, a subclone can be cultured from the corresponding streak plate after 24 h of incubation at 37 °C, 5 % CO_2 (Fig. 2f). If there are no pure positive but only mixed and negative reactions, pick several colonies from the streak plate made from a mixed colony and repeat the same screening procedure.

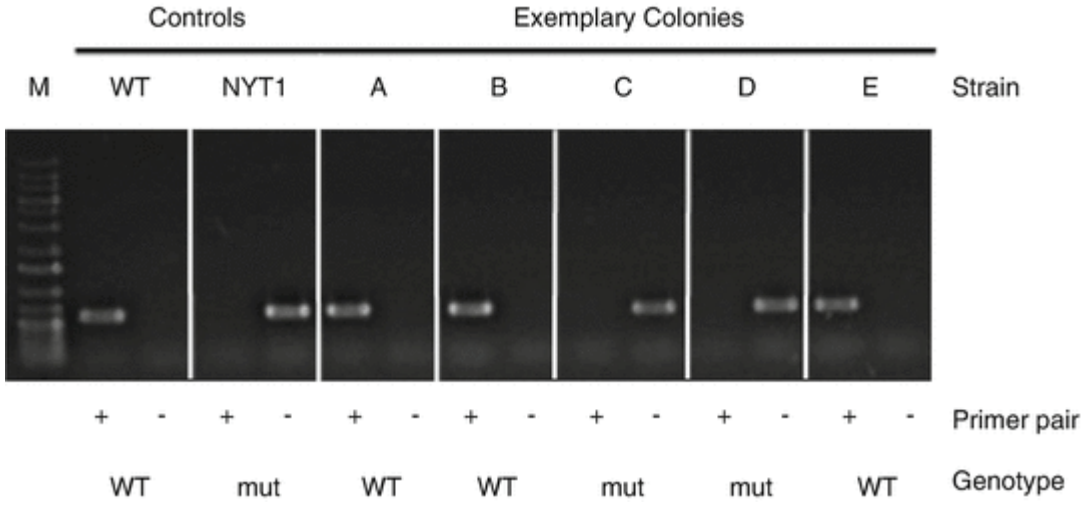

Fig. 3 Gel analysis of PCR products from exemplary colonies examined for transfer of suppressor single-base substitution in *S. pneumoniae* . +, primer pair specific for WT sequence; −, primer pair specific for mutant sequence. M, molecular weight standard; WT, parent strain; NYT1, suppressor strain; A–E, colonies tested; genotype, wild type (WT), or mutant (mut)

12. Repeat the screening procedure twice to allow for complete segregation of the mutan Prepare stocks by growing the selected bacteria in TSB overnight and then store the culture with 15 % glycerol at −20 °C or −80 °C (Fig. 2g).

13. A confirmatory PCR should be conducted once the stock is made to confirm that only the mutant allele is present. Final confirmation of the mutation in the target gene is done by sequencing (Fig. 2h).

3.3 **Markerless Transformation Protocol Using CSP in *S. pneumoniae***

1. Stock cultures prepared in TSB (OD_{550} 0.2) and stored with 15 % glycerol at −80 °C are diluted 1:100 in fresh TSB and incubated at 37 °C until OD_{550} 0.05.

2. To 1 mL of culture in a screw-cap tube, add CSP to a final concentration of 250 ng/mL, bovine serum albumin (BSA) to 0.04 %, $CaCl_2$ to 0.5 mM, 100 ng of donor amplicon DNA, and incubate for 80 min at 37 °C.

3. Prepare serial dilutions of the competent culture in TSB ; spread 100 μL of the following dilutions on TSB agar plates: 10^{-5}, 10^{-6}, and 10^{-7}. Incubate the plates for 16 h at 37 °C.

4. Design screening primers to distinguish donor from recipient alleles.

5. Pick 25 isolated colonies using sterile loops or needles; resuspend each in 10 μL in water. Prepare a streak plate with 2 μL of each suspension on a new TSB plate, and follow **steps 9–14** in Subheading 3.2.

3.4 Examples of Applications

In the applications of this method sketched in Fig. 2, three genes in *S. mutans* and one gene in *S. pneumoniae* were targeted for various alterations by use of sequences from GenBank accession numbers NC_004350 and NC_003098. Strains and specific primer sets for each case are listed in Tables 1, 2, 3, 4, and 5.

Table 1
List of strains

Name	Description	Source
Streptococcus mutans		
UA159	Wild-type reference strain	ATCC 700610
SM059	UA159, but p_{cipB}-luc::*spc*; Spc^R	[1]
SM068	UA159, p_{sigX}-luc; Spc^R	[1]
SM089	UA159, but Δ*comS*:: *ery*; Erm^R	[1]
SM091	SM068, but Δ*comS*:: *ery*; Spc^R Erm^R	[1]
SM134	SM089, but P_{cipB}-luc::*spc*; Erm^R Spc^R	SM089 × SM059

Name	Description	Source
SM177	SM068, but *comE* SigX-box inversion; Spc^R	SM068 × aRJ04
SM179	SM091, but *comE* SigX-box inversion; Spc^R Erm^R	SM091 × aRJ04
SM188	SM068, but c_105 1-bp substitution; Spc^R	SM068 × aRJ17
SM189	SM091, but ΔDR *comE*; Spc^R Erm^R	SM091 × aRJ18
SM190	SM134, but ΔDR *comE*; Erm^R Spc^R	SM134 × aRJ18
Streptococcus pneumoniae		
NYT1	CP2137, but Δ*comW*, *rpoD*-L363F; Sm^R Cm^R Kan^R Nv^R Erm^R Tc^R	[12]
CP2137	Δ*cps*, Δ*comA;* Sm^R Cm^R	[12]
CP2451	CP2137, but *rpoD*-L363F; Sm^R Cm^R	CP2137 × NYT1

Table 2
Primers for construction of *S. mutans* strains SM177 and SM179

Primer	Sequence (5′ to 3′)	Template	Amplicon product
FP916	GAGATGGGCTTTTTGGATGA	UA159	Segment 1
FP936	TCTACTAACTTAATAACCCTACTTATC TGCGAATAATATAATCAGATGATTAAGCAT		
FP917	TGCGGTCTATTGACCTCCTC	UA159	Segment 2
FP935	ATGCTTAATCATCTGATTATATTATTCGCAGA TAAGTAGGGTTATTAAGTTAGTAGA		
FP009	TATGGACCAAGAAATGCTGT	Segment 1+ Segment 2	Overlap PCR[a] 5757 bp—aRJ04
FP947	GCCCCCTTTATGGAACAAAT		
FP918	GCATAGGTGAGTCAAAGTGGTT	SM177 and SM179	194-bp allele-specific product
FP919	CTAACTTAATAACCCTACTTATCTGCGA		
FP920	AAGCAGTAATGCTAATGCTGTTAATC	UA159	347-bp allele-specific product
FP921	CTAACTTAATAACCCTACTTATCTATTCGCA		

Underlined letters represent inversion mutation

[a]Complementary primers FP935 and FP936 carry an inverted SigX-box of *comE*. FP009/FP947 were used to link segments 1 and 2, creating the final 5,757-bp amplicon aRJ04, which was used to transform strains SM068 and SM091, creating strains SM177 and SM179, respectively. Primer pairs FP918/FP919 and FP920/FP921 were used to detect the inversion in SM177 and SM179

Table 3
Primers for construction of *S. mutans* strain SM188

Primer	Sequence	Template	Amplicon product
FP894	TCCGGATGCAGAAGGTATTC	UA159	Segment 1
FP895	CAATAAAAGTTCTCACCCAATCTGGA		
FP896	TCCAGATTGGGTGAGAACTTTTATTG	UA159	Segment 2
FP897	CATCCTGCCGTTCCTATCAT		
FP937	TGTCCCGCTGGATACAGATT	Segment 1 + Segment 2	Overlap PCR[a]—aRJ17
FP938	TGTCCCGCTGGATACAGATT		

Primer	Sequence	Template	Amplicon product
FP898	GGTTGATTGGGTTTTTGTGG	UA159	444-bp allele-specific product
FP899	TTTTTATGCTTTTCAATAAAAGTTCTA		
FP900	CGGATTGGATTGGGAGACTA	SM188	681-bp allele-specific product
FP901	TTTTTATGCTTTTCAATAAAAGTTCTC		
FP902	CTCTAAGACTAATCCAGATTGGGTT	U159	364-bp allele-specific product
FP903	GCGAGTTTCAAAAAGGAAGC		
FP904	CTCTAAGACTAATCCAGATTGGGTG	SM188	552-bp allele-specific product
FP905	GGCAGACAGCTTCTTTGGTC		

Underlined letters represent the 1-base substitution

[a]Complementary primers FP895 and FP896 carry the single base substitution in *c105*. FP937/FP938 were used to overlap and link segments 1 and 2, creating the final amplicon aRJ17, which was used to transform strain SM068, creating mutant SM188. Primers FP898-FP905 were used to detect the base substitution in SM188

Table 4
Primers for construction of *S. mutans* strains SM189 and SM190

Primer	Sequence	Template	Amplicon product
FP1050	AATATAAAAGGGAGCGATGAAACTT	UA159	Segment 1
FP1051	GATAAGCAATAGATATAGCCTTCTTT GATCATGTTC		
FP1052	GAACATGATCAAAGAAGGCTATATCT ATTGCTTATC	UA159	Segment 2
FP1053	GTAGCTATTTTGTCCTAAACGGTCA		
FP1054	TGATTGTTTTTGTGGTATCTGCTAA	Segment 1 + segment 2	Overlap PCR[a]—aRJ18
FP1055	TTTACACAAGCTTTGGGAAAATAAG		
FP1056	CAACGGCTGATTAACAGAAAA	SM189 and SM190	309-bp product in UA159; but 272-bp product in mutants SM189 and SM190
FP968	TCATTCTAGTGATAATAAACATTTTGC		

[a]Complementary primers FP1051 and FP1052 carry the deleted direct repeat sequence of *comE*. FP1054/FP1055 were used to overlap and link segments 1 and 2, creating the final amplicon product aRJ18, which was used to transform strains SM091 and SM134, creating strains SM189 and SM190, respectively. FP1056 and FP968 were used to detect the deletion in mutants SM189 and SM190

Table 5
Primers used for the construction of *S. pneumoniae* mutant CP2451

Primer	Sequence (5′ to 3′)	Template	Amplicon product
YT30 YT31	GACAGGCTTTGAGTCTCTTGATGG CGGACGCTCAAACTTGGCTAATTC	NYT1	5.5 kb *rpoD*
YT49 YT51	CAAGTCGTAGCAAACCG**C** CACGGTAAGCACCTGAAAC	CP2137 (control) NYT1	500-bp WT allele specific product
YT50 YT51	CAAGTCGTAGCAAACCG**T** CACGGTAAGCACCTGAAAC	CP2137 NYT1 (control)	500-bp mutant allele specific product

Underlined letters represent the 1-base substitution

3.4.1 Example 1. Eight-Basepair Inversion

The method can be used to invert small sequences in the genome. In this example, the objective was to investigate a promoter region of *comE* putatively recognized by SigX [3] in *S. mutans* UA159, by making an 8-bp inversion. The steps of Subheading 3.1 were followed for the creation of the amplicon that was used to transform SM068 and SM091 (p*sigX*::luc reporter derivatives of UA159) into SM177 and SM179 (*see* Table 1; Fig. 4).

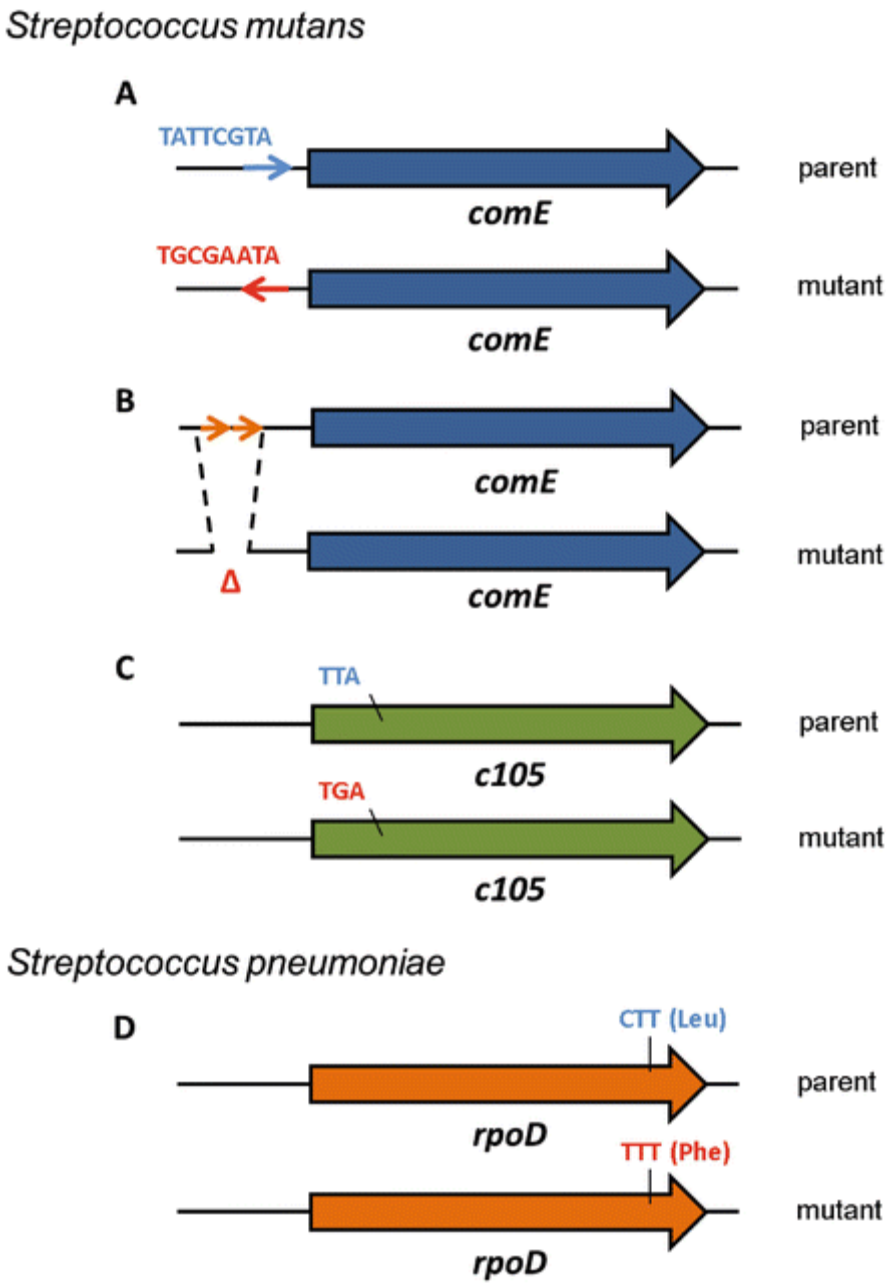

Fig. 4 Genomic changes made in four application examples of direct editing. In *S. mutans* , (**a**) inversion of a *comE* SigX-box, (**b**) deletion of two direct repeats, and (**c**) substitution of a single base to introduce a stop codon within gene smut_orf_c105. In *S. pneumoniae* , (**d**) substitution of a single base causing a Leu363 → Phe replacement in RpoD

Complementary primers (P2 and P3) carrying the inversion were designed and respectively matched by primers located in the flanking regions (P1 and P4) to create two segments of 4.3 kb (P1/P2) and 4 kb (P3/P4).

Nested primers (P5 and P6) 5.7 kb apart (*see* Fig. 4) were used to connect the two segments with overlapping PCR, creating a final amplicon product having the mutation in its center.

This final product was used to transform *S. mutans* (*see* Subheading 3.2).

Twelve colonies were screened. Seven were mixed; four were pure mutant clones (33 %). Pure mutant colonies were isolated, re-screened, and stocked as strains SM177 and SM179 (Table 1).

3.4.2 Example 2. Thirty-Nine-Basepair Deletion

To investigate the function of two direct repeats located close to the putative promoter region of *comE* (Fig. 2) in *S. mutans* UA159, primers for PCR (P2 and P3) were designed to flank the region selected for deletion, and respectively matched by primers located in distal flanking regions (P1 and P4) to create two segments of 2.9 kb (P1/P2) and 2.8 kb (P3/P4).

Nested primers (P5 and P6) 5.1 kb apart were used to connect the two segments with overlapping PCR, creating the final amplicon product containing a central deletion.

The final amplicon product was used to transform *S. mutans* strains SM091 and SM134. Among 16 colonies screened, 4 were mixed (25 %), and 12 were negative. To allow segregation, these 4 colonies were grown in TSB the next day, plated, re-selected, and re-screened twice in order to isolate the pure mutant clone, creating strains SM189 and SM190.

3.4.3 Example 3. Single-Base Substitution in *S. mutans*

To investigate the function of an open-reading frame (smut_orf_1_105) that overlaps SMU_60 in *S. mutans* UA159, a base substitution was designed to create a stop codon in the former ORF but retain unaltered translation of the latter ORF.

Complementary primers (P2 and P3) carrying the single-base substitution were designed and respectively matched by primers located in the flanking regions (P1 and P4) to create two segments of 3.9 kb (P1/P2) and 3.8 kb (P3/P4).

Nested primers (P5 and P6) 7.5 kb apart were used to connect the two segments by overlapping PCR, creating a final amplicon product having the mutation in its center. The final amplicon product was used to transform *S. mutans* UA159 (*see* **Note 13**).

The screening primers in this case required use of touchdown PCR to achieve discriminatory specificity (*see* **Note 14** and **15**). The annealing temperature was reduced gradually from 68 to 63 °C during the first 15 cycles and maintained at 63 °C during remaining cycles. Otherwise the program was the same as described above (Subheading 3.2, **step 10**). A mixed colony was re-streaked, and a verified pure mutant subclone was retained as strain SM188 (Table 1). The mutation was confirmed by DNA sequencing.

3.4.4 Example 4: Single-Base Substitution in *S. pneumoniae*

Single-amino acid substitutions in the *S. pneumoniae* primary sigma factor (RpoD) can bypass the need for the critical ComW component during transformation [12]. To investigate the effect of the corresponding single-base substitution, a 5.5-kb region around *rpoD* of strain NYT1 (Table 1) was amplified using primers YT30 and YT31 (Table 5), centered on the mutant base (Fig. 4d).

Mutant sequences were amplified, purified, and transformed into *S. pneumoniae* strain CP2137. Using a 69 °C annealing temperature during the colony-screening PCR, the screening yielded 50 % pure transformants based on amplification from the primer set complementary to the mutant sequence.

One colony was streaked out and 10 subclones from this streak were again tested by PCR. Although 9 of 10 colonies again showed the mutant sequence, one showed the WT sequence, indicating the need to re-streak and pick isolated colonies. A single subclone with the mutant sequence was reconfirmed by DNA sequencing and named CP2451.

4 Notes

1. Other suitable agar plates with media supporting growth of streptococci such as Brain Heart Infusion (BHI) or Todd Hewitt Broth (THB) may also be used. Antibiotics were used at the following concentrations: kanamycin (Kan), 500 μg/mL; erythromycin (Erm), 10 μg/mL; spectinomycin (Spc), 500 μg/mL.

2. CDM is stored in closed bottles at 4 °C for up to 4 weeks.

3. XIP or CSP peptides received as crude desalted product of >80 % purity are routinely highly active.

4. It is important to adjust the annealing temperature for each pair of primers when using this kit.

5. To increase the efficiency of the PCR reaction with nested primers, it is important to use less than 20 ng of fragments a and b in a 50-μL reaction.

6. The most important factor here is to grow pre-cultures from fresh colonies that were plated just a day before. Streak the strain on a TSB plate and incubate overnight at 37 °C in CO_2. Next day, resuspend a group of colonies in 10 mL liquid CDM medium (initial OD_{600} = 0.05 to 0.1), grow approximately 3 h in a capped tube or in CO_2 until mid-log phase (OD_{600} = 0.5). Add 1/5 volume of glycerol, prepare aliquots of 1 mL, and store at −80 °C. We observed a remarkable increase in transformation efficiency—from ~7 % to >30 % - when we started preparing pre-cultures this way.

7. Dilution of pre-cultures may be optimized down to 1:100,000 to increase the proportion of transformants.

8. We found 75 ng to be a saturating amount of donor amplicon. Transformation assays were usually conducted in closed 1.5-mL Eppendorf tubes. It is not recommended to increase the time of incubation with the donor amplicon beyond 3 or 4 h.

9. Efficiency varies depending on the locus of the amplicon, for unknown reasons.

10. Given that transformation efficiencies are high, it is also possible to screen fewer colonies. We recommend using 24 as a good resource to estimate the efficiency of the experiment and also ensure recovery of a mutant.

11. If the transformation has lower efficiency, a group of colonies can be collected for PCR screening. Once a group containing a positive colony is identified, it can be streaked again to isolate individual subclones for screening.

12. In addition, as a second step of screening, a second pair of primers, P9 and P10 can be designed with at least one of them in, e.g. the deleted region, therefore binding to the DNA of the parent but not to the mutant. This allows for an independent confirmation of the deletion (Fig. 2e).

13. If the nature of the mutation provides fragments with the same MW in the mutant and the parent allele, two pairs of primers can be designed here, the first pair with one of the primers binding only to the parent, and the second with one of the primers binding exclusively to the mutant. It is convenient, however, to design these for fragments with different MW for differential identification.

14. When designing codon changes it is important to check for rare codon usage [14].

15. Touchdown PCR involves decreasing the annealing temperature in small increments and provides specificity by favoring the specific base pairing between primer and template [15].

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