Genome Editing by Natural Genetic Transformation in *Streptococcus mutans*

D. A. Morrison^{2,3}, R. Khan¹, R. Junges¹, H. A. Åmdal¹, F. C. Petersen¹

¹ Department of Oral Biology, Faculty of Dentistry, University of Oslo, Norway; ² Department of Biological Sciences, College of Liberal Arts and Sciences, University of Illinois at Chicago, USA

³ **Corresponding Author:** D. A. Morrison, Room 4102 MBRB, 900 South Ashland Ave, Chicago, IL USA 60607

E-mail addresses:

Donald Morrison	DAMorris@uic.edu
Rabia Khan	rabia.khan@odont.uio.no
Roger Junges	roger.junges@odont.uio.no
Heidi Åmdal	h.a.amdal@odont.uio.no
Fernanda Petersen	f.c.petersen@odont.uio.no

Keywords: pheromone, competence, natural transformation, markerless mutagenesis

Abbreviations

CDM, chemically defined medium

CSP, competence-stimulating peptide

XIP, SigmaX-inducing peptide

PCR, polymerase chain reaction

1. Introduction

Genome editing has gained currency as a shorthand to represent a family of recently developed strategies for directed mutation, in which DNA disruption by clustered regularly interspaced short palindromic repeats (CRISPR) nucleases at specific sites within eukaryotic genomes is coupled with recombinational repair of the damage to introduce arbitrarily designed genetic changes at chosen sites. Equivalent repair-initiating damages are also introduced at unique sites in eukaryotic genomes by use of zinc finger nucleases (ZFNs) or the class of transcription-activator-like effector nucleases (TALENs). DNA selected or designed and produced *in vitro* is then introduced as a substrate for the repair process. The resulting specificity of target for the introduced sequences creates dramatic advantages over traditional transgenic approaches, which introduce trans DNA at random or poorly controlled sites, as emphasized by the new moniker. Strictly speaking, genome editing has long been practiced routinely as a tool of bacterial genetics, by use of natural bacterial genetic transformation as the facilitating and targeting mechanism, and by manipulation of donor DNA *in vitro* to provide arbitrary sequence alterations. In this case, targeting damage is not required, as competence development creates a specialized cell equipped with two dozen proteins that together accomplish targeted gene replacement by extracting long multi-gene segments of ssDNA from native DNA molecules, preserving them after transport, and efficiently inserting them with high precision at matching resident sequences (Johnston et al., 2014). No special targeting devices are needed, because the donor DNA itself is used by the native inducible recombination system for precise targeting anywhere in the genome. Because yields of recombinants among recipient cells are often in the .01–1% range or lower, most such designs have retrieved rare recombinants by incorporation of a readily selectable marker linked to the desired mutation.

A drawback to the use of selective markers for gene inactivation is the introduction of new genes unrelated to the mutant phenotype of interest, risking phenotypic effects of the inserted genes and accumulation of multiple irrelevant new genes during successive steps of mutagenesis. To avoid such drawbacks, various strategies have been developed for achieving gene deletion or modification without introduction of permanent selective markers unrelated to the desired mutation in both the archaea and the bacteria. Some use the Cre-*loxP* system for excision of the unwanted marker (Banerjee and Biswas, 2008; Fontaine et al., 2010b; Leibig et al., 2008). Others use a recombinant plasmid for insertion by single homologous cross-over followed by a different

crossover to remove unwanted plasmid elements (Biswas et al., 1993; Keller et al., 2009; Lipscomb et al., 2011; Olson and Lynd, 2012). A third approach is illustrated by an elegant cloning-independent counter selection approach using transformation with a *pheS-ermAM* cassette to create markerless mutations by counter selection with p-Cl-Phe. The two-step procedure inserts a highly expressed mutant *pheS* allele that confers p-Cl-Phe sensitivity at a target site using selection for erythromycin resistance, then uses a second transformation with a mutant construct carrying the desired new allele to replace the temporary *pheS-ermAM* cassette by double-crossover recombination and selecting for loss of *pheS* (Xie et al., 2011). Using the high specificity of natural genetic transformation, many versions of these two steps have been developed, especially by relying on a variety of strong counter selections at the second step, based on initial incorporation of two linked genes – one to provide selection for insertion, and a second to allow selection for the excision event (Debowski et al., 2012; Farkas et al., 2012; Thiel et al., 2014; Zhang and Whitaker, 2012). However, the design of suitable selectable and counter-selectable markers can be complex. In a fourth, more general strategy, co-transformation by a mutagenic PCR amplicon donor and a counter selectable plasmid selects for the minority of competent cells, which are subsequently screened by colony PCR for incorporation of the desired mutation; removal of the T^S plasmid then allows recovery of an unmarked mutant (Biswas et al., 2007).

The general principle underlying these methods has been to combine recombination events of low probability but high precision with strong selection to achieve predictable genetic rearrangements at a practical frequency. As each such selection strategy has potential disadvantages, we sought to learn whether the efficient and precise targeted recombination reaction of natural genetic transformation itself could be utilized for direct one-step mutagenesis while bypassing selection steps entirely. All systems of natural genetic transformation described in bacteria and archaea to date transfer genetic information from extracellular DNA molecules into the recipient genome in two steps, following a pattern characterized in detail for *Streptococcus pneumoniae* and *Bacillus subtilis*. First, a transport apparatus extracts ssDNA segments of arbitrary length from DNA encountered by the cell, starting at a single-strand break introduced at random (Lacks et al., 1967; Morrison and Guild, 1973). Although ssDNA is subject to attack by intracellular nucleases, competent cells are in fact strongly protective of the imported ssDNA. Although short fragments are frequently degraded, long fragments are quite thoroughly protected (Morrison and Guild, 1972). The second step, homologous recombination, then produces long

stretches of heteroduplex containing integrated donor nucleic acid (Gurney and Fox, 1968). In some species, 100% of cells in a laboratory culture become highly competent at the same time; in others, although only a minority becomes competent, these few can individually be highly competent. The efficiency of gene replacement depends on the length of donor DNA strands, reaching 50% or more for pure donor with lengths above 4 kb and diminishing to zero below 300 bp (Cato and Guild, 1968). Thus, when combined with use of PCR to prepare unique donor fragments of chosen design, the high efficiency of recombination in natural transformation should in principle make direct genome editing practical. The power of large donor DNA fragments for gene replacement by natural transformation was illustrated recently in two contrasting circumstances: in *S. pneumoniae*, where all cells are simultaneously highly competent, and in *Vibrio cholerae*, where few cells are competent (Dalia et al., 2014). Observation of a high proportion of double transformations in *V. cholerae* demonstrated that once formed, DNA uptake and incorporation can be highly efficient in competent cells of this species .

The human oral commensal *S. mutans*, first described in 1924 (Clarke, 1924), is associated with caries and appears to have evolved in specific adaptation to human sugar-rich diets (Cornejo et al., 2013; Loesche, 1986). Discovery of natural genetic transformation in *S. mutans* in 1981 (Perry and Kuramitsu, 1981) has provided the basis for many tools for its genetic manipulation, facilitating study of its metabolism and cariogenic potential. While low and variable levels of transformation in routine laboratory culture made selective markers especially important in developing these tools, recent advances in characterization of the control of competence development in this species have improved available levels of competence significantly. First, the peptide pheromone XIP (*sigX*-inducing peptide) can be used to intervene at the heart of competence regulation, by directly provoking a high rate of synthesis of SigX, the central competence regulator (Mashburn-Warren et al., 2010). Second, competence in this species is reported to be bimodal in rich media, but unimodal in peptide-free chemically defined media (Lemme et al., 2011; Reck et al., 2015; Son et al., 2015; Son et al., 2012). This immediately suggests a chemically defined culture medium as especially suitable for development of strategies for direct genome editing, which would depend on efficient gene incorporation by most or all cells exposed to DNA. Although improved levels of transformation in chemically defined media have been reported (Shah and Caufield, 1993), a direct measurement of maximal replacement rates is not available. Here we report that in *S. mutans* the competent state can be more like that of *S.*

pneumoniae than that of *V. cholerae*, coupling a high fraction of competent cells and a high rate of gene replacement, and show that XIP-induced competence in *S. mutans* can provide a practical route to convenient direct genome editing, without resort to linked selective markers.

2. Methods

2.1. Bacterial strains and culture media

The *S. mutans* strains used in the study are listed in Table 1. SM068, a *sigX* reporter derivative of strain UA159, was used as the recipient for most transformation assays. All strains were cultured in tryptic soy broth (TSB) or a chemically defined medium (CDM) (Vanderijn and Kessler, 1980). CDM was assembled weekly from the stable stock solutions described previously (Chang et al., 2011). Media were solidified when needed with 1.5% Bacto agar (Becton, Dickinson and Company). Antibiotics were used at the following concentrations: kanamycin (Kan), 500 µg/mL; erythromycin (Erm), 10 µg/mL; spectinomycin (Spc), 500 µg/mL. The mature competence pheromone CSP18 (Petersen et al., 2006) (NH₂-SGSLSTFFRLFNRSFTQA-COOH) was obtained) as a custom synthetic peptide (GenScript Corporation, NJ), at 98% purity and stored as sterile 1 mM solution in water at -20°C. The alternative competence pheromone XIP (NH₂-GLDWWSL-COOH; 98% purity; GenScript) was reconstituted with 20 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich), to which 1 mL distilled water was added to give a final concentration of 10 mM.

2.2 Luciferase reporter assays

For detection of luciferase activity, precultures of the *PsigX*-luciferase strain SM068 grown in CDM to an optical density at 600 nm (OD₆₀₀) of 0.5 were prepared as described below for the transformation assays. For inoculation, pre-cultures were diluted 10-fold in CDM or TSB in the presence or absence of 1 µM XIP or 0,25 µM CSP, respectively. Aliquots of 100 µl were then distributed among the wells of black 96-well microtiter plates with transparent bottoms (Nunc Thermo Scientific). Wells with CDM or TSB medium without the inoculum were included as blanks. A volume of 10 µl of a 1 mM d-luciferin solution (Synchem, Felsberg-Altenberg, Germany) was then added to each well, and the cultures were incubated at 37°C in air. Relative

luminescence units (RLU) and OD₆₀₀ were measured at various time intervals during growth by reading the plates in a multi-detection microplate reader (Synergy HT; BioTek, Winooski, VT).

2.3. *Marked mutant strain construction*

Three new marked deletion strains (SM033, SM045, and SM156) were constructed by PCR-ligation mutagenesis (Lau et al., 2002), using 1-kb fragments amplified from a genomic template up- and down-stream of a target gene, and a third fragment containing a selective antibiotic marker, amplified from a plasmid. After digestion with restriction enzymes and ligation by T4 DNA ligase (New England Biolabs), the three-component ligation product was amplified and transformed into *S. mutans* UA159 by culturing cells with CSP18 as described previously (Petersen et al., 2006). The structure of the mutant targeted locus in a selected transformant was verified by amplification of the predicted junction products. Details of individual constructions and a list of primers designed for each purpose are given in Supplemental Table 1.

2.4. *Markerless mutant strain construction*

To construct the markerless mutant SM177, with inversion of a putative SigX-box promoter upstream of the bacteriocin regulatory gene *comE*, fragments of 4.3 kb and 3 kb were amplified from strain UA159 with the primer pairs FP916-FP936 and FP935-FP913, respectively. Primers FP935 and FP936 were complementary to each other, with a central region comprising the inverted SigX-box sequence. The overlapping sequences were used for fusion of the two PCR products, in a ligase-free PCR reaction that used less than 5 ng of each of the two amplified sequences and the nested primers FP009 and FP910, to produce a conjoint amplicon of 5757 bp. After transformation of competent cells of strain SM068, as described in section 2.4, colonies from TSB plates were screened by PCR (section 2.5), colony-purified, and re-screened to identify a clone containing the inversion but no WT SigX-box. Similar designs and procedures yielded strains SM179, SM189 and SM190, as detailed in Supplemental Table 1.

2.5 *Transformation assays*

The standard transformation was done as previously described, with slight modifications (Desai et al., 2012). Pre-cultures were prepared by transferring fresh colonies grown on TSB agar

plates to liquid CDM, followed by growth at 37°C to an OD₆₀₀ of 0.5. The pre-cultures were then stored at -80°C with 15 % glycerol until use. On the day of transformation, the pre-cultures were diluted 1:10 in CDM and incubated at 37°C until reaching the exponential phase at OD₆₀₀ 0.1. Unless specified otherwise, the cultures were then exposed to 0.1 - 1 µg/mL DNA and 1 µM XIP for 2-3 h at 37°C in closed tubes. Portions of the culture were then spread on TSB plates with relevant antibiotic, if any. After 15-36 hours at 37°C in 5% CO₂, colonies were counted. For routine quantitation of transformants, 3-µL or 20-µL drops from serial dilutions were spotted on the surface of selective or non-selective TSB/agar plates, allowed to dry, and inspected for small colonies after 20-36 h incubation at 37°C in 5% CO₂. Spots with 3 – 100 colonies were counted under a dissecting microscope.

2.6. Colony genotyping assays

Colonies on non-selective plates were screened by PCR using primers specific, respectively, for the donor and recipient alleles at targeted loci. Briefly, a 10-µL PCR reaction was set up with 0.06 µL of each primer, 0.2 µL dNTP mix (10mM), 1 µL 10x buffer, 1 µL MgCl₂ (25mM), 0.2 µL TrueStart Taq polymerase (Thermo Scientific; 5U/µL) and 7.5 µL of nuclease-free distilled water. Each colony was picked with a sterile pipette tip for transfer to the PCR-mix and a TSB stock plate. PCR cycles were: 3 min at 95°C; 25 repetitions of 30 s at 95°C, 30 s at 55°C, and 50 s at 72°C; and a final extension for 5 min at 72°C.

2.7. Preparation of genomic donor DNA

Genomic donor DNA was extracted from strains according to Lin Tao (personal communication), as described previously (Desai et al., 2012).

2.8. Preparation of donor DNA amplicons

Primers used for amplification of donor DNA from existing mutants are listed in Supplemental Table 1 and corresponding amplicons are described in Table 2. The PCR reactions contained: 10 µL 5 × Q5 buffer, 1 µL 10 mM dNTP mix, 0.25 µL of 100 µM primers, 50 ng template, and 1 unit of Q5 DNA polymerase (New England BioLabs) in 50 µL total volume. Initial denaturation at 98 °C for 30 s was followed by 5 cycles of 98 °C for 10 s, 54 °C annealing for 30

s, and 72 °C extension for 90 s, 25 cycles of 98 °C for 30 s, 65 °C annealing for 30 s, and 72 °C extension for 2 min, and a final 72 °C extension for 5 min. Amplicons used for fusion PCR were purified using the GeneJET PCR Purification Kit (Thermo Scientific). The fusion overlap PCR reactions generating mutagenic donor amplicons consisted of 100 ng of each of the two amplicons to be fused, 10 µL 5 × Q5 reaction buffer, 0.3 µL of 100 µM “nested” primers, 1 µL 10 mM dNTP mix, and 0.5 units of Q5 DNA polymerase in 50 µL total volume. The PCR program was set to: 98 °C initial denaturation for 30 s; 30 cycles of denaturation at 98 °C for 10 s, 62 °C annealing for 30 s, and 72 °C extension for 30 s/kb of product; and a final extension at 72 °C for 5 min. The amplified products were used directly for transformation.

3. Results and Discussion

3.1. Optimal conditions for high efficiency of gene replacement in *S. mutans*

We explored the use of the SigmaX-inducing peptide, XIP, to induce competence in the chemically defined medium, CDM, because this medium supports unimodal competence in *S. mutans* in response to XIP (Lemme et al., 2011; Reck et al., 2015; Son et al., 2015; Son et al., 2012). A second reason was that the XIP receptor, ComR, is the proximal regulator of *sigX*, suggesting that intervention at this stage of the competence signaling pathway would likely be the least complex strategy for obtaining optimal competence development (Mashburn-Warren et al., 2010). Because the bacteriocin-regulating pheromone known as CSP is often used to stimulate development of competence in *S. mutans* in rich media (Li et al., 2001), a direct comparison of the transcriptional responses to CSP vs. XIP was carried out to learn which condition for competence development might be superior when the goal is a maximal rate of gene replacement or alteration. To compare induction of competence using XIP in CDM vs. CSP in a richer medium, a single culture was split and incubated in parallel in the two media with respective pheromone peptides (Fig. 1). Monitoring of luminescence from a *psigX* reporter showed that although growth rates were higher in TSB than in CDM, *sigX* expression reached higher levels and persisted for a longer time in CDM, explaining the long period of active DNA uptake described below (section 3.2).

To ask whether any density dependence remained when synthetic XIP was used to bypass the ComRS quorum-sensing step in competence regulation, CDM cultures were treated with XIP at various low cell densities. The quality of competence, assessed as transformants per mL achieved during a 2-h exposure to XIP and DNA, was independent of culture density over a wide range of initial culture densities (0.001 to 0.02) and densities at the start of DNA exposure (0.009 to 0.11) (Fig. 2). In contrast to early reports for *S. mutans* that a specific culture density is optimal for competence development (Lindler and Macrina, 1986), this result indicates that the provision of synthetic XIP short-circuits much density dependence, as is the case for supplying pheromone peptides to other naturally transformable streptococci (Fontaine et al., 2010a; Havarstein et al., 1995).

3.2. Competence of *S. mutans* in CDM is long-lasting

To optimize the protocol for transformation further, we explored the kinetics of competence development in CDM cultures treated with XIP. Competence, measured directly as the momentary rate of DNA uptake, followed a characteristic long time course (Fig. 3A). During the first 20 min after XIP introduction, almost no cells exhibited competence. There followed a period of 80 min during which capacity for DNA uptake constantly increased. Beyond 80 min, cells remained highly competent for at least two additional hours. Environmental pH is a critical factor in *S. mutans* competence, which is optimal at values close to pH 7, and reduced under acidic conditions (Guo et al., 2014; Son et al., 2015). Monitoring of pH revealed that the initial pH 7 of our cultures remained unchanged for at least 7 h, which may explain in part the long period of sustained competence. This prolonged state of competence indicated that the best yield of transformants might be obtained by extended exposure to donor DNA. Indeed, introduction of donor DNA at either 50 min or 150 min after XIP led, in either case, to continuous DNA uptake at a constant rate during at least 200 min (Fig. 3B).

3.3. High efficiency of incorporation of large unique donor DNA amplicons

A critical parameter in determining the yield of recombinants during transformation is expected to be the complexity of donor DNA. For example, a 7-kb PCR product carrying a gene of interest is ~300-fold less complex than genomic DNA from the same donor strain, and thus contains much less irrelevant competitor DNA material than a genomic DNA preparation. The

value of using such enriched donors is illustrated for *S. mutans* in experiments collected in Fig. 4, where cultures in CDM were treated with XIP and a 6.3-kb PCR product carrying a kanamycin-resistance insert (aRJ102) at final concentrations ranging from 0.3 to 150 ng per mL. Direct comparison of CFU on Kan medium vs unselective medium showed that maximal transformant yields of approximately 40% were achieved with amplicon concentrations of 75 ng mL⁻¹ or above (Fig. 4).

In this example, the entire *dexA* gene was replaced by non-homologous DNA, in the form of the Kan^R marker. Although these events were monitored by use of the selective marker, the efficiency of replacement was so high as to make the selection step appear unnecessary, so long as some tool is available to screen a few dozen colonies for incorporation of the new material. Since any mutation can in principle be detected by appropriately designed PCR reactions, it seemed likely that arbitrary base changes could be readily introduced in this way using a markerless large donor DNA construct.

3.4. Introduction of an eight-base inversion

As a first test of such direct, ‘markerless,’ genome editing and to investigate a promoter region putatively recognized by a known regulator, we designed a mutation in which an 8-bp motif suspected of being a SigX-dependent promoter for the gene *comE* (Reck et al., 2015) was substituted by its reverse complement sequence. Assembly of a 5757-bp donor amplicon by overlap extension PCR and design of primer pairs to screen clones after transformation are described in Section 2.4. After extended incubation of this donor amplicon with a competent culture in CDM, two dozen colonies on TSB were screened by PCR for presence of the donor allele; screening two dozen subclones from positive cultures was sufficient to recover pure transformant clones (data not shown). Optimization of incubation time in the presence of XIP and donor DNA, resulted in up to 66 % colonies with only the mutant allele (Fig. 5).

3.5. Efficiency depends on size of donor fragments

While the yield of recombinants from amplicon donors can be very high, it is expected to depend on donor size. In the related competence system of *S. pneumoniae*, for example, recombinant yield for sheared genomic DNA donors is roughly proportional to length of DNA

donor molecules within the range of 1 – 10 kb (Cato and Guild, 1968) and the yield of recombinants from donor amplicons varies over a 10,000-fold range when homologous arms vary between 100 and 2000 bp (Lau et al., 2002). To explore the upper range of this relationship for *S. mutans*, donor amplicons of different sizes, but directing the same allelic change, were constructed by amplification from genomic templates by use of symmetrically placed primers designed at different distances either from the small inversion mutation described above or from a Kan insert in gene SMU.60. Figure 6, comparing gene replacement rates for the amplicon donors with flanking homology ranging from 0.5-3 kb, indicates that a similarly strong size dependence does hold for *S. mutans*, with efficiency increasing little if at all for extension of flanking homology arms above the size of 2 kb, but decreasing severely below that size. Very similar size dependence was seen for both types of allele: the small sequence change and the larger gene insertion. Figures 6 and 7 both indicate that a maximal rate of gene replacement is reached with 2 to 3 kb flanking regions of homology; it is not known why *dexA::kan* and 8-bp inversion donors gave higher transformation efficiency than the SMU.60::*kan* donor, but the difference was reproducible in different experiments.

Results of additional transformation trials indicated that targeted mutagenesis by this strategy could be achieved routinely. Using three recipient derivatives of UA159, two donor DNA amplicons (8 and 6 kb), in 24 trials on five different days with DNA at 0.15 – 1 µg/ml, cultures at OD 0.04 to 0.1, and exposure times of 2.5 – 6 h, transformation frequencies were observed ranging from 17% to 60% (Supplemental Table 2).

3.6. *Extended incubation increases recombinant yield and allows segregation of pure transformant clones*

The chaining growth habit of streptococci is likely to ensure that colonies recovered unselectively soon after transformation are mixed – containing both recombinants and parental cells. However, as gene replacements were very frequent, it appeared possible that pure transformant colonies might be generated at a significant rate during moderately extended incubation in CDM. In two trials, we tested for such clones using various lengths of incubation with DNA and XIP exposure, by testing individual colonies by PCR for presence of donor and recipient alleles (Fig. 5, Fig. 7). Pure clones were found at a significant frequency after incubations as short as 3 h.

321
322 *3.7. Additional examples of genome sequence editing without use of selective resistance markers.*

323 Illustrating the utility of direct genome editing, two additional markerless mutations were
324 designed and created using this method (for design details, see Supplemental Table 1). First, a
325 single-base replacement was made in the putative ORF c105 (Human Oral Microbiome Database
326 annotation; www.homd.org) to create a stop codon, without making any change to the translation
327 product of an overlapping ORF, SMU.60. A T61178G substitution retained Val75 in SMU.60
328 (GTT > GTG) but replaced Leu16 in the overlapping ORF c105 by a stop codon (TTA > TGA) in
329 strain SM068, to create strain SM188. Primer pairs to verify the mutation were designed so that
330 the 3' terminal base of one of them corresponded to the mutated base. Touch-down PCR starting
331 with high annealing temperatures that were decreased for each PCR cycle ensured specific
332 amplification. Second, a deletion of a putative direct-repeat binding site for ComE was made by
333 removal of 39 bp upstream of the *comE* gene in strains SM091 and SM134, creating strains
334 SM189 and SM190. In this case, the mutated region was distinguished from the wild-type by the
335 reduced size of the product amplified by PCR in the mutant. Figure 8 summarizes the range of
336 structural changes that we have obtained using direct genome editing.

3.8. Applications to other strains of *S. mutans* and to other streptococcus species

The results of these initial experiments on the use of pure-gene donors suggest that direct genome editing in *S. mutans* strain UA159 will be powerful and practical. Already, 10-50% of CFU were regularly been found to carry a donor cassette gene that had been provided in 5-7-kb pure gene fragments, as prepared by PCR amplification. Similarly high replacement rates were obtained for the markerless mutation design that included an 8-bp inversion of a SigX-box in the promoter regions. Other designs included a 39-bp deletion and a 1-bp substitution (Fig. 8). Variations in the dose of donor DNA for *S. mutans* revealed high levels of transformation with saturating concentrations above 75 ng mL⁻¹ for ~4-kb donors. A possible drawback to the method for single-base replacement may arise from activity of Hex repair systems. The mismatch repair system Hex modulates the yield of transformants for 1-base changes in *S. pneumoniae*, and a similar effect may be in operation in *S. mutans* (Claverys and Lacks, 1986). If so, it can be expected that certain 1-base substitutions may be disfavored by as much as two- to ten-fold, but that larger alterations would escape such repair. A second possible drawback arises from the possibility of errors during PCR amplification of the flanking DNA critical for precise targeting. However, modern generations of proof-reading polymerases are so accurate as to minimize this concern.

Since many isolates of *S. mutans* are known to produce and respond to XIP (Khan, 2015), and since the sequence of *comS* is widely, perhaps universally, conserved within this species, the genome editing method described here may have wider application, beyond the reference strain UA159 that was used in these studies. Preparation of donor DNA is likely to present few technical hurdles. However, application of this method outside UA159 may face a variety of challenges rooted in the biology of the recipient competent cells. In certain media, for example, strain UA159 develops competence in only a subset of cells within a culture (Lemme et al., 2011; Reck et al., 2015; Son et al., 2015; Son et al., 2012). In strains that transform poorly, but have a minority of fully competent cells, one can use two different pieces of donor DNA - one at an innocuous site with selection and a second DNA fragment carrying the desired mutation. Then the screening can be focused on selected transformants for the first marker, to identify the desired un-marked chromosomal mutation.

Finally, as similarly high direct editing rates were reported for competent cultures of *S. pneumoniae*, where 5-kb amplicons were used to make 1-bp substitutions (Tovpeko and Morrison,

2014), it is likely that this direct editing strategy would be useful in other streptococcal species where competence development can be optimized. Such optimization will be facilitated by the recent recognition that all streptococci share the SigX regulator and either of two types of upstream pheromone signals -- CSP or XIP (Johnston et al., 2014). Experience over two decades has shown that these readily available peptide pheromones can short-circuit uncharacterized environmental factors to achieve very high rates of transformation.

3.9. Conclusion

Until the era of inexpensive whole-genome synthesis arrives, each strategy for targeted mutagenesis will represent a balance of choices among available tools and paths, choices made to minimize cost while maximizing precision, efficiency, and speed. The strategy described here for *S. mutans* can be applied directly to the widely studied reference strain UA159 and has the advantage of extreme simplicity, requiring construction of only one synthetic donor amplicon and a single transformation step, followed by a simple PCR screen among a few dozen clones to identify the desired mutant. The strategy depends on optimizing both partners in natural genetic transformation, donor DNA and competent recipient cells. The donor is provided as a specially designed pure gene amplicon, carrying the mutant sequence centrally located between flanking segments of homology of at least two kb, which ensure efficient and precise integration by the recombination machinery specific to competent cells. The recipients are highly competent cells, maintained in a persistent state of competence by treatment with the 7-residue synthetic competence XIP pheromone peptide in a chemically defined peptide-free medium.

4. Acknowledgments.

This work was supported in part by the National Science Foundation under grant no. MCB-1020863, and by the Faculty of Dentistry, University of Oslo. We thank Kunal Desai for assistance with exploratory experiments.

References

- Banerjee A, Biswas I (2008). Markerless multiple-gene-deletion system for *Streptococcus mutans*. *Applied and Environmental Microbiology* 74(7):2037-2042.
- Biswas I, Gruss A, Ehrlich SD, Maguin E (1993). High-efficiency gene inactivation and replacement system for gram-positive bacteria. *Journal of Bacteriology* 175(11):3628-3635.
- Biswas I, Drake L, Johnson S, Thielen D (2007). Unmarked gene modification in *Streptococcus mutans* by a cotransformation strategy with a thermosensitive plasmid. *Biotechniques* 42(4):487-490.
- Cato A, Jr., Guild WR (1968). Transformation and DNA size: I. Activity of fragments of defined size and a fit to a random double cross-over model. *Journal of Molecular Biology* 37(1):157-178.
- Chang JC, LaSarre B, Jimenez JC, Aggarwal C, Federle MJ (2011). Two group A streptococcal peptide pheromones act through opposing Rgg regulators to control biofilm development. *Plos Pathogens* 7(8):e1002190.
- Clarke JK (1924). On the bacterial factor in the etiology of dental caries. *British Journal of Experimental Pathology* 5(3):141-147.
- Claverys JP, Lacks SA (1986). Heteroduplex deoxyribonucleic acid base mismatch repair in bacteria. *Microbiological Reviews* 50(2):133-165.
- Cornejo OE, Lefebure T, Bitar PDP, Lang P, Richards VP, Eilertson K *et al.* (2013). Evolutionary and population genomics of the cavity causing bacteria *Streptococcus mutans*. *Molecular Biology and Evolution* 30(4):881-893.
- Dalia AB, McDonough E, Camilli A (2014). Multiplex genome editing by natural transformation. *Proceedings of the National Academy of Sciences of the United States of America* 111(24):8937-8942.
- Debowski AW, Gauntlett JC, Li H, Liao TT, Sehna M, Nilsson HO *et al.* (2012). Xer-cise in *Helicobacter pylori*: One-step transformation for the construction of markerless gene deletions. *Helicobacter* 17(6):435-443.
- Desai K, Mashburn-Warren L, Federle MJ, Morrison DA (2012). Development of competence for genetic transformation of *Streptococcus mutans* in a chemically defined medium. *Journal of Bacteriology* 194(15):3774-3780.

Farkas J, Stirrett K, Lipscomb GL, Nixon W, Scott RA, Adams MWW *et al.* (2012). Recombinogenic properties of *Pyrococcus furiosus* strain COM1 enable rapid selection of targeted mutants. *Applied and Environmental Microbiology* 78(13):4669-4676.

Fontaine L, Boutry C, de Frahan MH, Delplace B, Fremaux C, Horvath P *et al.* (2010a). A novel pheromone quorum-sensing system controls the development of natural competence in *Streptococcus thermophilus* and *Streptococcus salivarius*. *Journal of Bacteriology* 192(5):1444-1454.

Fontaine L, Dandoy D, Boutry C, Delplace B, de Frahan MH, Fremaux C *et al.* (2010b). Development of a versatile procedure based on natural transformation for marker-free targeted genetic modification in *Streptococcus thermophilus*. *Applied and Environmental Microbiology* 76(23):7870-7877.

Guo Q, Ahn SJ, Kaspar J, Zhou X, Burne RA (2014). Growth phase and pH influence peptide signaling for competence development in *Streptococcus mutans*. *Journal of Bacteriology* 196(2):227-236.

Gurney T, Fox MS (1968). Physical and genetic hybrids formed in bacterial transformation. *Journal of Molecular Biology* 32(1):83-100.

Havarstein LS, Coomaraswamy G, Morrison DA (1995). An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* 92(24):11140-11144.

Johnston C, Campo N, Berge MJ, Polard P, Claverys JP (2014). *Streptococcus pneumoniae*, le transformiste. *Trends in Microbiology* 22(3):113-119.

Keller KL, Bender KS, Wall JD (2009). Development of a markerless genetic exchange system for *Desulfovibrio vulgaris Hildenborough* and its use in generating a strain with increased transformation efficiency. *Applied and Environmental Microbiology* 75(24):7682-7691.

Khan R, Rukke HV, Ricomini AP, Fimland G, Arntzen MO, Thiede B *et al.* (2012). Extracellular identification of a processed type II ComR/ComS pheromone of *Streptococcus mutans*. *Journal of Bacteriology* 194(15):3781-3788.

Khan R (2015). Genetic transformation in *Streptococcus mutans*: Role of CSP and XIP signaling pheromones. (Doctoral thesis). Oslo, Norway, University of Oslo.

Lacks S, Greenber.B, Carlson K (1967). Fate of donor DNA in pneumococcal transformation. *Journal of Molecular Biology* 29(3):327-347.

Lau PCY, Sung CK, Lee JH, Morrison DA, Cvitkovitch DG (2002). PCR ligation mutagenesis in transformable streptococci: application and efficiency. *Journal of Microbiological Methods* 49(2):193-205.

- Leibig M, Krismer B, Kolb M, Friede A, Gotz F, Bertram R (2008). Marker removal in staphylococci via Cre recombinase and different lox sites. *Applied and Environmental Microbiology* 74(5):1316-1323.
- Lemme A, Grobe L, Reck M, Tomasch J, Wagner-Dobler I (2011). Subpopulation-Specific transcriptome analysis of competence stimulating peptide induced *Streptococcus mutans*. *Journal of Bacteriology* 193(8):1863-1877.
- Li YH, Lau PCY, Lee JH, Ellen RP, Cvitkovitch DG (2001). Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *Journal of Bacteriology* 183(3):897-908.
- Lindler LE, Macrina FL (1986). Characterization of genetic transformation in *Streptococcus mutans* by using a novel high-efficiency plasmid marker rescue system. *Journal of Bacteriology* 166(2):658-665.
- Lipscomb GL, Stirrett K, Schut GJ, Yang F, Jenney FE, Scott RA *et al.* (2011). Natural competence in the hyperthermophilic archaeon *Pyrococcus furiosus* facilitates genetic manipulation: Construction of markerless deletions of genes encoding the two cytoplasmic hydrogenases. *Applied and Environmental Microbiology* 77(7):2232-2238.
- Loesche WJ (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiological Reviews* 50(4):353-380.
- Mashburn-Warren L, Morrison DA, Federle MJ (2010). A novel double-tryptophan peptide pheromone controls competence in *Streptococcus* spp. via an Rgg regulator. *Molecular Microbiology* 78(3):589-606.
- Morrison DA, Guild WR (1972). Transformation and deoxyribonucleic acid size: extent of degradation on entry varies with size of donor. *Journal of Bacteriology* 112(3):1157-1168.
- Morrison DA, Guild WR (1973). Breakage prior to entry of donor DNA in pneumococcus transformation. *Biochimica Et Biophysica Acta* 299(4):545-556.
- Olson DG, Lynd LR (2012). Transformation of *Clostridium thermocellum* by electroporation. *Methods in Enzymology* 510(317-330).
- Perry D, Kuramitsu HK (1981). Genetic transformation of *Streptococcus mutans*. *Infection and Immunity* 32(3):1295-1297.
- Petersen FC, Fimland G, Scheie AA (2006). Purification and functional studies of a potent modified quorum-sensing peptide and a two-peptide bacteriocin in *Streptococcus mutans*. *Molecular Microbiology* 61(5):1322-1334.

Reck M, Tomasch J, Wagner-Dobler I (2015). The Alternative Sigma Factor SigX Controls Bacteriocin Synthesis and Competence, the Two Quorum Sensing Regulated Traits in *Streptococcus mutans*. *PLoS Genetics* 11(7):e1005353.

Shah GR, Caufield PW (1993). Enhanced transformation of *Streptococcus mutans* by modifications in culture conditions. *Analytical Biochemistry* 214(1):343-346.

Son M, Ghoreishi D, Ahn SJ, Burne RA, Hagen SJ (2015). Sharply Tuned pH Response of Genetic Competence Regulation in *Streptococcus mutans*: a Microfluidic Study of the Environmental Sensitivity of comX. *Applied and Environmental Microbiology* 81(16):5622-5631.

Son MJ, Ahn SJ, Guo Q, Burne RA, Hagen SJ (2012). Microfluidic study of competence regulation in *Streptococcus mutans*: environmental inputs modulate bimodal and unimodal expression of comX. *Molecular Microbiology* 86(2):258-272.

Thiel A, Michoud G, Moalic Y, Flament D, Jebbar M (2014). Genetic manipulations of the hyperthermophilic piezophilic Archaeon *Thermococcus barophilus*. *Applied and Environmental Microbiology* 80(7):2299-2306.

Tovpeko Y, Morrison DA (2014). Competence for Genetic Transformation in *Streptococcus pneumoniae*: Mutations in σ^A Bypass the comW Requirement. *Journal of Bacteriology* 196(21):3724-3734.

Vanderijn I, Kessler RE (1980). Growth characteristics of group A streptococci in a new chemically defined medium. *Infection and Immunity* 27(2):444-448.

Xie ZJ, Okinaga T, Qi FX, Zhang ZJ, Merritt J (2011). Cloning-Independent and Counterselectable markerless mutagenesis System in *Streptococcus mutans*. *Applied and Environmental Microbiology* 77(22):8025-8033.

Zhang CY, Whitaker RJ (2012). A broadly applicable gene knockout system for the thermoacidophilic archaeon *Sulfolobus islandicus* based on simvastatin selection. *Microbiology* 158(1513-1522).

548 **Table 1. Strains used.**

Name	Description	Source
MW01	UA159, but $\Delta comR::CAT$; Cm ^R	(Mashburn-Warren et al., 2010)
MW04	UA159, but $\Delta oppD::spc$; Spc ^R	(Mashburn-Warren et al., 2010)
UA159	Wild-type	ATCC 700610
SM045	UA159, but $\Delta dexA::kan$; Kan ^R	UA159 x TPLP*
SM059	UA159, but p_{cipB} -luc:: spc ; Spc ^R	(Khan et al., 2012)
SM067	UA159, but $\Delta oppD::spc$; Spc ^R	UA159 x MW04
SM068	UA159, p_{sigX} -luc; Spc ^R	(Khan et al., 2012)
SM089	UA159, but $\Delta comS::ery$; Erm ^R	(Khan et al., 2012)
SM091	SM068, but $\Delta comS::ery$; Sp ^R Erm ^R	(Khan et al., 2012)
SM156	UA159, but $\Delta smu_{60}::Kan$; Kan ^R	UA159 x TPLP*
SM134	SM089, but P_{cipB} -luc:: spc ; Erm ^R Spc ^R	SM089 x SM059
SM177	SM068, but $comE$ SigX-box inversion; Spc ^R	SM068 x aRJ04
SM179	SM091, but $comE$ SigX-box inversion; Spc ^R Erm ^R	SM091 x aRJ04
SM188	SM068, but c_105 1-bp substitution; Spc ^R	SM068 x aRJ17
SM189	SM091, but $\Delta DR comE$; Spc ^R	SM091 x aRJ18
SM190	SM134, but $\Delta DR comE$; Spc ^R	SM134 x aRJ18

549 *TPLP, three-piece ligation product; details available in Supplemental Table 1

550

551 **Table 2. Donor DNA amplicons**

ID	Marker	Source	Total Length, bp	Left arm	Cassette or Nucleotide substitutions, bp	Right arm, bp
aRJ01	Δ SMU.60:: <i>kan</i>	SM156	8033	4075	1038	2920
aRJ02	Δ dexA:: <i>kan</i>	SM045	6311	2574	884	2853
aRJ04	p-comE SigX-box _{INV}	Assembly or SM177	5757	2893	Inv 8 bp	2856
aRJ05	p-comE SigX-box _{INV}	SM177	8294	3987	Inv8 bp	4299
aRJ06	p-comE SigX-box _{INV}	SM177	6017	3030	Inv 8 bp	2979
aRJ07	p-comE SigX-box _{INV}	SM177	4037	1936	Inv 8 bp	2093
aRJ08	p-comE SigX-box _{INV}	SM177	2001	1054	Inv 8 bp	939
aRJ09	p-comE SigX-box _{INV}	SM177	1014	515	Inv 8 bp	491
aRJ12	p-comE SigX-box _{INV}	SM177	4158	3987	Inv 6 bp	163
aRJ13	Δ SMU.60:: <i>kan</i>	SM156	7077	3119	1038	2920
aRJ14	Δ SMU.60:: <i>kan</i>	SM156	5282	2142	1038	2102
aRJ15	Δ SMU.60:: <i>kan</i>	SM156	3076	1016	1038	1022
aRJ16	Δ SMU.60:: <i>kan</i>	SM156	2149	549	1038	562
aRJ17	c_105 1-bp substitution	SM188	7575	3618	1	3956
aRJ18	Δ DR <i>comE</i>	SM189	5116	2556	39	2560

552

553

Figure Legends

Figure 1. Comparison of *sigX* expression profiles in CDM and TSB. Strain SM068 was grown in 100 μ L volumes of CDM or TSB in a 96-well plate in ambient air at 37°C with 1 μ M XIP (Black) or 250 nM CSP-18 (grey), respectively. (A) Culture density and (B) *sigX* expression (*sigX* reporter luminescence measured as relative light units, RLU) were monitored periodically. The results are representative of two independent experiments.

Figure 2. Density-independent competence in CDM. Transformation of dilute CDM cultures was measured following XIP treatment. A frozen stock of SM068 in CDM was used to inoculate 12 CDM cultures at various initial optical densities. OD₆₀₀ was measured for each culture after 4 h, when SM089 genomic DNA (1 μ g/ml) and XIP (1 μ M) were added. Transformants (grey) and total CFU (black) were determined after 2 hs further incubation.

Figure 3. Persistent competence in *S. mutans* treated with XIP. Three cultures of UA159 growing in CDM were supplemented with XIP (3 μ M) at OD 0.07. (A) One culture was repeatedly assayed for competence by exposing 1-ml samples to 10 μ g/ml of MW01 DNA for 5 min, treated with DNase I in TSB at 37 C for 2 hrs to stop further uptake and allow expression of the Cm^R phenotype. (B) The other two cultures were supplemented with MW01 DNA at 10 μ g/ml after 50 (grey) or 150 min (black) with XIP. Each was subsequently assayed for accumulation of Cm^R transformants at 5- or 10- min intervals during 100 min at 37 C. Standard deviations for triplicate plate counts were 35%, 34%, and 30% respectively.

Figure 4. Transformation as a function of amplicon DNA concentration. Strain SM068 was transformed with the 6.3-kb amplicon donor aRJ02 in 200- μ L volumes of CDM with 1 μ M XIP for 3 h. Amplicon concentrations varied from 0.3 to 150 ng mL⁻¹. The % of CFU containing a KanR transformant was determined by plating on TSB and TSB Kan agar. The results are averages from 3 to 4 replicates. Bars represent standard errors.

Figure 5. Segregation of pure transformant clones. Ex-transformation colonies growing on nonselective medium were analyzed for recipient and donor alleles by PCR. Plating on TSB was

done after incubation of SM068 with the 5.8-kb amplicon aRJ04 and XIP for 3 – 6 hs. Each bar displays results for 12 colonies. **Neg**, only recipient allele observed (grey) ; **Pure**, only donor allele observed (maroon); **Mixed**, both alleles observed (pink). (B) Gel image of exemplary **Pure**, **Neg**, and **Mixed** colonies. M, Gene RulerTM 1kb DNA ladder. Control templates: INV, amplicon aRJ04, with SigX-box inversion; WT, UA159 gDNA. Screening primers were: FP919-FP920 (347-bp, for SigX-box inversion), and FP918-FP921 (194 bp, for WT).

Figure 6. Dependence of gene replacement efficiency on amplicon size. Transformation of SM068 in CDM with XIP by markerless SigX-box inversion fragments from strain SM177 (aRJ05-aRJ09), constructed with 0.5- to 4-kb arms, or by SMU.60-*kan* amplicons from strain SM156 (aRJ13 – aRJ16) with 0.5- to 3-kb arms at 150 or 300 ng/mL. % of Kan-selected transformants is indicated at left (SM156 amplicons), and unselected donor alleles at right (PCR screen; SM177 amplicons).

Figure 7. Effect of donor DNA length on segregation of pure recombinants. Strain SM068 was incubated with XIP and 0.1 nM DNA donor fragments carrying the SigX-box inversion for 3.5 h at 37°C. Dilutions (10^{-7}) were spread on TSB plates. For each donor, 24 colonies were screened by PCR to detect the donor allele, using primer pairs as in Fig. 5. Colony lysates giving a donor PCR band were re-analysed for recipient allele, to allow characterization as pure recipient, pure mutant (maroon) donor allele, or mixed (pink). Amplicon donors of defined size were prepared from strain SM177 as amplicons aRJ05-09 and aRJ12.

Figure 8. Summary of mutation designs. Target regions in each panel is at bottom, donor DNA amplicon at top, with desired alteration indicated in **green**. (A) SMU.60 deletion, (B) *dexA* deletion, (C) inversion of the putative SigX-box upstream of *comE* , (D) single base mutation in c105 (E) deletion of the ComE direct repeat (DR) binding site upstream of *comE*. Represented genes: *kan*, kanamycin resistance; SMU.60, alkylation repair protein; *dexA*, extracellular dextranase; *comE*, response regulator of the ComDE two-component signal transduction system; c105, putative ORF overlapping the 3-end of SMU.60.

Figure 1

(A)

(B)

Figure 2

Figure 3

(A)

(B)

Figure 4

Figure 5

(B)

Figure 6

Figure 7

Figure 8

(A)

(B)

(C)

(D)

(E)

Graphical Abstract