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Cloning-independent plasmid construction for genetic studies in streptococci

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ABSTRACT

Shuttle plasmids are among the few routinely utilized tools in the *Streptococcus mutans* genetic system that still require the use of classical cloning methodologies and intermediate hosts for genetic manipulation. Accordingly, it typically requires considerably less time and effort to introduce mutations onto the *S. mutans* chromosome than it does to construct shuttle vectors for expressing genes in trans. Occasionally, shuttle vector constructs also exhibit toxicity in *Escherichia coli*, which prevents their proper assembly. To circumvent these limitations, we modified a prolonged overlap extension PCR (POE-PCR) protocol to facilitate direct plasmid assembly in *S. mutans*. Using solely PCR, we created the reporter vector pZX7, which contains a single minimal streptococcal replication origin and harbors a spectinomycin resistance cassette and the *gusA* gene encoding β-glucuronidase. We compared the efficiency of pZX7 assembly using multiple strains of *S. mutans* and were able to obtain from 5×10^3 to 2×10^5 CFU/μg PCR product. Likewise, we used pZX7 to further demonstrate that *Streptococcus sanguinis* and *Streptococcus gordonii* are also excellent hosts for cloning-independent plasmid assembly, which suggests that this system is likely to function in numerous other streptococci. Consequently, it should be possible to completely forgo the use of *E. coli–Streptococcus* shuttle vectors in many streptococcal species, thereby decreasing the time and effort required to assemble constructs and eliminating any toxicity issues associated with intermediate hosts.

1. Introduction

Genetic research of streptococci has benefited tremendously from the efficient natural competence ability of many of these species coupled with the increasingly diverse palette of genetic tools that have been adapted for use in these organisms. Among the human oral streptococci, *Streptococcus mutans* has the most sophisticated genetic system. Various types of gene mutations can be engineered in a surprisingly short time frame using a variety of antibiotic resistance cassettes (Kuramitsu, 1993; Kuramitsu, 2003; Russell, 1994) or via several markerless mutagenesis systems (Atlagic et al., 2006; Banerjee and Biswas, 2008; Merritt et al., 2007; Xie et al., 2011). Multiple shuttle vectors are also available for complementation (Biswas et al., 2008; Chen and LeBlanc, 1992; Chen et al., 2011; Macrina et al., 1982). Likewise, these same tools are routinely employed to introduce numerous types of reporter genes to assay gene expression or for in situ imaging applications (Goodman and Gao, 1999; Honeyman et al., 2002; Hudson and Curtiss, 1990; Kreth et al., 2004; Yoshida and Kuramitsu, 2002). As is, the *S. mutans* genetic toolbox is already more akin to that of a model organism (Lemos et al., 2013). However, unlike *Escherichia coli* genetic research, genetic work in *S. mutans* still requires intermediate hosts to clone constructs. Classical cloning methodologies utilizing restriction enzymes and ligases are also frequently the most frustrating and labor intensive steps during strain construction and can form significant obstacles to progress when the cloned DNA fragments exhibit toxicity in *E. coli*. Consequently, it has been one of our longstanding goals to improve the *S. mutans* genetic system to the point where we no longer require such cloning methodologies or intermediate hosts. We have recently made significant progress toward this goal by creating a cloning-independent markerless mutagenesis system for *S. mutans* (Xie et al., 2011). Using only overlap extension PCR, we are now able to create unlimited chromosomal deletions, point mutations, truncations, reporter gene insertions, etc. all within a wild-type parent background and free of antibiotic resistance cassettes or scars on the chromosome. This single system has already greatly diminished the need for *E. coli* in the majority of our *S. mutans* genetic work. A prominent exception is plasmid construction. Often times it is necessary to express a gene in trans, which necessitates cloning constructs onto *E. coli–Streptococcus* shuttle vectors. Since streptococcal promoters are typically active in *E. coli*, cloned genes tend to be highly expressed due to the plasmid copy number and frequently exhibit toxicity (Warren et al., 2007). Consequently, certain constructs may be difficult or impossible to assemble.

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Recently, two separate cloning-independent plasmid construction methodologies have been reported for use in *E. coli* and *Bacillus subtilis* (van den Ent and Lowe, 2006; You et al., 2011). These systems both utilize similar prolonged overlap extension PCR (POE-PCR) approaches to introduce inserts into target vectors. The insert is first PCR amplified with primers that add stretches of vector complementarity to the insert amplicon. Next, the insert amplicon is mixed in an equimolar concentration with either a circular plasmid or PCR amplified plasmid in a final primerless PCR reaction. For this reaction, the complementary sequences engineered into the insert amplicon serve as the primers for the final PCR step. Thus, additional primers are not required for polymerization. Each round of denaturation and annealing in the PCR reaction allows the terminal ends of the insert amplicon to hybridize to the target vector, which then facilitates ligation and amplification by the polymerase during the extension step (Bryksin and Matsumura, 2010; You et al., 2011). The continual cycling in the PCR reaction ultimately generates a series of linear concatemers consisting of variable numbers of repeated subunits of vector + insert and will appear as high molecular weight smears in agarose gels after electrophoresis. Interestingly, if these linear concatemers are transformed, they are actually recircularized intracellularly into stably replicating plasmids, presumably due to recombination between the numerous repeats of vector + insert within the molecules (You et al., 2011). Given our previous success with overlap extension PCR for mutagenesis, we were curious whether PCR generated concatemers would function for plasmid assembly in *S. mutans*. Here, we demonstrate that this approach is highly efficient in multiple strains of *S. mutans* as well as in *Streptococcus sanguinis* and *Streptococcus gordonii*. These results suggest that numerous streptococcal species should be able to directly serve as hosts for vector construction, which eliminates the requirement for classical cloning methodologies, shuttle vectors, and intermediate hosts during plasmid assembly.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All *Streptococcus* strains were grown anaerobically (in an atmosphere consisting of 85% N2, 10% CO2, and 5% H2) at 37 °C. For natural transformation experiments, cells were maintained in Todd-Hewitt medium (Difco) supplemented with 0.3% (wt/vol) yeast extract (THYE). For the selection of antibiotic-resistant colonies in *S. mutans*, BHI plates were supplemented with spectinomycin (Sigma) (1000 μg ml^{-1}). For the selection of antibiotic-resistant colonies in *S. sanguinis* or *S. gordonii*, BHI plates were supplemented with spectinomycin (500 μg ml^{-1}). To visualize β-glucuronidase activity in transformants, BHI plates were supplemented with 0.75 mM 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glu) (Biosynth International Inc). The competence-stimulating peptide used for *S. mutans* transformation (CSP-Sm) was custom-synthesized by Anaspec, while the competence-stimulating peptide used for *S. sanguinis* transformation (CSP-Ss) was kindly provided by Dr. J. Kreth.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference
Strains		
UA140	Wild-type *Streptococcus mutans*	(Qi et al., 2001)
UA159	Wild-type *Streptococcus mutans* reference strain	(Ajdic et al., 2002)
GS5	Wild-type *Streptococcus mutans*	(Biswas and Biswas, 2012)
ATCC 25175	Wild-type *Streptococcus mutans*	
SK36	Wild-type *Streptococcus sanguinis*	(Xu et al., 2007)
DL1	Wild-type *Streptococcus gordonii*	(Vickerman et al., 2007)
PRgusA	UA140 *hdrR*$_P$::*gusA*, Δ*hdrRM*, markerless	Unpublished data
Plasmids		
pZX7	pVA380::*hdrR*$_P$::*gusA*, spec^r	This study
pDL278	*E. coli–Streptococcus* shuttle vector, spec^r	This study
pFW5	Streptococcal suicide vector, spec^r	(Podbielski et al., 1996)
pET44b	*E. coli* expression vector, amp^r	

2.2. Cloning-independent plasmid construction in Streptococcus strains

A similar protocol was employed here as has been described in detail for use in *E. coli* and *B. subtilis* (You et al., 2011) and is briefly summarized. Firstly, linear DNA fragments of vector and insert were generated by PCR with primers adding >40 bp of complementarity to the amplicon termini. Secondly, concatemers were generated by prolonged overlap extension PCR (POE-PCR) using the amplicons generated in the first PCR reaction as the template and primers. Finally, the POE-PCR products (concatemers) were directly transformed into *Streptococcus* strains via natural transformation. The primers used in this study are shown in Table 2. Phusion DNA polymerase (New England BioLabs) was used for the PCR steps in this study. Generation of the vector and insert amplicons in the first step PCR was performed according to the manufacturers' instructions as follows: 10 μL 5× Phusion buffer, 1 μL 10 mM dNTP mix, 1 μL of 10 μM forward and reverse primer, <10 ng template, and 1 unit of enzyme were mixed in 50 μL total volume. The reaction was cycled using an initial denaturation of 98 °C for 30 s followed by 30 cycles of 98 °C denaturation for 10 s + annealing at primer Tm for 15 s + 72 °C extension for 2 kb/min and a final 72 °C extension for 5 min. The PCR amplicons were subsequently purified using the Qiagen PCR purification kit. The second step PCR generating the concatemers was assembled as follows. Each 50 μL reaction contained: 100 ng insert amplicon, equimolar concentration of vector amplicon, 10 μL 5× Phusion reaction buffer, 1 μL 10 mM dNTP mix, and 1 unit of Phusion DNA polymerase. The PCR program was set according to the following: 98 °C initial denaturation for 30 s, 25 cycles of denaturation at 98 °C for 10 s + 65 °C annealing for 15 s + 72 °C extension for a period based on the size of the final plasmid (2 kb/min.), and a final extension at 72 °C for 5 min.

2.3. Natural transformation of S. mutans UA140, UA159, GS5, 25175

S. mutans strains were cultivated overnight in THYE. The following day, stationary phase cultures were diluted 1:20 in THYE and incubated at 37 °C until the OD_{600} reached 0.2–0.3 (about 2–3 h). 1 μg of transforming DNA (concatemer PCR) and 1 μg ml^{-1} final concentration CSP-Sm were added into each 500 μl cell culture and incubated for another 2 h. After the incubation period, cells were diluted appropriately before plating a portion on selective BHI plates and incubating at 37 °C until colonies were visible. Depending upon the efficiency of plasmid construction in a given strain, it was necessary to plate varying dilutions of the transformation reactions to obtain the desired range of 200–500 CFU/plate.

2.4. Visualization of plasmids extracted from S. mutans

Six UA159 POE-PCR transformants were randomly selected and inoculated into separate tubes containing 3 mL BHI + spectinomycin. Stationary phase cultures were resuspended in 500 μL buffer (10 mM pH 7.5 Tris-Cl + 1 mM EDTA + 10 mg/mL lysozyme). The cells were incubated for 4 h at 37 °C. Following the incubation period, the plasmids were extracted using the Qiagen Plasmid Miniprep kit beginning with the lysis step in the manufacturer's protocol. 500 μL buffer P2 and 700 μL buffer N3 were used for alkaline lysis and neutralization, respectively. Clarified lysates from each culture were loaded into six spin columns and washed according to the

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