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RESEARCH

Improving the Efficiency of Transposon Mutagenesis in *Salmonella* Enteritidis by Overcoming Host-Restriction Barriers

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Abstract Transposon mutagenesis using transposome complex is a powerful method for functional genomics analysis in diverse bacteria by creating a large number of random mutants to prepare a genome-saturating mutant library. However, strong host restriction barriers can lead to limitations with species- or strain-specific restriction-modification systems. The purpose of this study was to enhance the transposon mutagenesis efficiency of *Salmonella* Enteritidis to generate a larger number of random insertion mutants. Host-adapted Tn5 DNA was used to form a transposome complex, and this simple approach significantly and consistently improved the efficiency of transposon mutagenesis, resulting in a 46-fold increase in the efficiency as compared to non-adapted transposon DNA fragments. Random nature of Tn5 insertions was confirmed by high-throughput sequencing of the Tn5-junction sequences. The result based on *S.* Enteritidis in this study should find broad applications in preparing a comprehensive mutant library of other species using transposome complex.

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Introduction

Transposon mutagenesis using a transposon–transposase complex (transposome) has been used in diverse bacterial species to create random transposon mutants [1–14]. The mutant libraries thus prepared are a critical resource for functional genomics analysis of bacteria for gene identification. When the goal is to screen the mutant library for phenotypes of interest, it is desirable to create a comprehensive library that covers the entire genome. This usually means thousands to hundreds of thousands of independent random insertions, depending on the genome size [11, 15]. However, the efficiency of mutagenesis is often inhibited by the host restriction-modification system in the bacterial cells [16–19]. When EZ:Tn5^{TM} Transposome complex (Epicentre Biotechnologies, Madison, WI, USA) is used to transform *Salmonella* Typhimurium (ST) strains, we routinely obtain 10^4–10^5 mutants per electroporation. However, with *Salmonella* Enteritidis (SE) strains, the efficiency of mutagenesis drops by greater than 100-fold and only 10^2 transformants are produced per electroporation.

S. Enteritidis is one of the top five non-typhoidal serovars causing human salmonellosis infections with wide-ranging reservoirs and the serovar most commonly associated with eggs and egg products. Many reported human infection cases of salmonellosis were frequently linked to SE. In the European Union, SE was the cause of 60 % of *Salmonella* outbreaks in 2009 [20]. In the United States, SE was responsible of almost one fifth of all *Salmonella* reported cases in 2009, and in 2010, a half billion eggs were recalled as the result of an outbreak caused by SE

[21]. Our knowledge on the genetic tropism of this serovar as the distinctive predominance on eggs is limited [20–22]. To identify genes essential for SE to be associated with poultry and their products, specifically eggs, we are initially required to generate a comprehensive random insertions library to screen the whole genome.

An earlier attempt to overcome the restriction of transforming plasmids DNA to *Salmonella* strains was made by Tsai and others using a ST strain with defective restriction system (JR501) to modify plasmids for *Salmonella* compatibility [23]. Afterward, the strain JR501 and similarly constructed strains have been used into various genetic studies of *Salmonella* [24–35].

Edwards et al. [36] reported a simple method to overcome host restriction barriers to protect the exogenous DNA introduced into SE against host restriction systems, thus increasing transformation efficiency. This technique involves heat treatment during preparation of the competent cells to temporarily inactivate or reduce the activity of the DNA restriction systems within the host cells and enable successful transformation. In this study, we used host-adapted transposon DNA to improve the efficiency of transposon mutagenesis and compared the result with that obtained with heat-inactivation method for temporary inactivation of the host restriction barrier of SE using the EZ: Tn5^{TM} Transposome complex (Epicentre Biotechnologies).

In a study by Colegio et al., a host-adapted Tn552 fragment in *Campylobacter jejuni* was used to form a complex with transposase [37]. When the complex was used to transform *C. jejuni*, it efficiently increased transposition efficiency 30–80 times as compared with that without host adaptation and readily generated 2.9×10^3–7.7×10^3 mutants/electroporation. Therefore, we hypothesized that a similar strategy can increase transformation efficiency when transposon mutagenesis is performed for SE strain using EZ: Tn5^{TM} Transposome complex.

Materials and Methods

Bacterial Strains and Culture Conditions

EZ-Tn5^{TM} pMOD-6 < KAN-2/MCS > transposon construction vector (Epicentre Biotechnologies, Madison, WI, USA) was maintained in TOP10 *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) in Luria–Bertani (LB) broth with 100 % glycerol at −80 °C. *Salmonella* Enteritidis PT13A (National Veterinary Services Laboratories, Ames, IA) was used for transposon mutagenesis library construction. Luria–Bertani agar media (BD Difco, Sparks, MD) were first used for growth of all frozen bacterial cultures with 24 h of incubation at 37 °C. SOC media (**S**uper **o**ptimal broth with carbon **c**atabolite repression, generally glucose) (Invitrogen, Carlsbad, CA, USA) were used immediately for recovering the cells after electro-transformation. As suitable, the following antibiotics were added to the media, novobiocin (25 μg/ml) (No) and kanamycin (50 μg/ml) (Km).

Electro-Transformation

S. Enteritidis PT13A had been prepared for electroporation according to a previously described protocol with few modifications [38]. Briefly, a single colony of bacterial culture from LB agar was inoculated into 10 ml of 2X YT broth (BD Difco, Sparks, MD) with novobiocin and grown at 37 °C overnight with vigorous shaking. Subsequently, 100 μl of overnight culture was re-inoculated into 15 ml of fresh 2X YT broth without salt (16 g/L tryptone, 10 g/L yeast extract) and incubated at 37 °C for 3–4 h to reach mid-log corresponding to an approximate OD_{600} of 0.6 (0.5–0.7). Instantly, cultures were chilled on ice followed by centrifugation, and pellets were kept on ice throughout the procedures for electro-competent cell preparation. Cells were washed five times in ice-cold sterile ddH_2O water and finally re-suspended in 70 μl of ice-cold sterile 10 % glycerol. The prepared electro-competent cells were used instantaneously and were mixed with DNA. After incubation in ice for 10 min, the cells were then electroporated at 2,450 kV with 2-mm cuvettes for 5–6 ms using ECM 399 Electroporation System (Harvard Apparatus, Holliston, MA), subsequently incubated in SOC for 1–1.5 h at 37 °C and plated on LB media with appropriate antibiotics.

Heat-Inactivation Method

Briefly, the above preparation protocol of electro-competent cells was used with slight modification. Mid-log cultures of SE PT13A were incubated at 50 °C for 25 min and then kept on ice throughout the preparation of electro-competent cells [38]. Subsequently, cells were immediately used for electroporation with 2 μl of EZ: Tn5^{TM} Transposome complex. To recover the transposon mutants after performing each electro-transformation, we immediately added 500 μl of pre-warmed SOC medium to the reaction and incubated it at 37 °C for 1 h with vigorous shaking and then enumerated on LB agar plates supplemented with kanamycin (50 μg/ml). After incubation at 37 °C for 18 h, the colony forming units (number of mutants) were enumerated.

Host-Adapted DNA Method

A strategy to overcome the host restriction barrier is to adapt the exogenous transposon DNA to the host cells before the introduction as demonstrated previously with

slight modification [37]. To test the hypothesis, we performed transposon mutagenesis of SE PT13A strain using EZ: Tn5™ transposon that had been replicated in SE PT13A wild type (adapted DNA) or *E. coli* Top10 (non-adapted DNA; negative control). Initially, we used 1 µl of EZ-Tn5™ pMOD-6 < KAN-2/MCS > transposon construction vector (Epicentre Biotechnologies, Madison, WI, USA) to transform SE PT13A (adapted transposon DNA) or *E. coli* Top10 (non-adapted transposon DNA, as control) by electroporation. The transformants were inoculated on LB agar plates supplemented with kanamycin (50 µg/ml) for each of SE PT13A or *E. coli* Top10 and incubated overnight at 37 °C.

One transformant colony resistant to kanamycin from each SE PT13A or *E. coli* Top10 was inoculated into LB broth containing 50 µg/ml of kanamycin, and incubated overnight at 37 °C. Plasmid DNA was purified using QIAprep Miniprep kit (Qiagen, Valencia, CA) from the overnight cultures, and the concentration and purity were checked with a NanoDrop 1,000 spectrophotometer (Thermo Scientific, Wilmington, DE). Restriction enzyme digestion of plasmid DNA was performed with PvuII-HF following the manufacture instruction (New England Biolabs, Ipswich, MA) to release EZ: Tn5 fragments of 1,117 bp long.

These fragments were carefully extracted from gels using a QIAquick gel extraction kit (Qiagen, Valencia, CA) without exposing the transposon DNA to ultraviolet light during visualization. Afterward, the purified transposon DNA was checked for concentration and purity as indicated above. The Tn5 transposome complex was produced by mixing 2 ul of the Tn5 transposon DNA with concentration of approximately 200 ng/µl, 4 ul EZ-Tn5™ Transposase (Epicentre Biotechnologies, Madison, WI, USA), and 2 ul of 100 % sterile glycerol in magnesium minus milieu as directed by the manufacturer's manual. Subsequently, the reaction was incubated at room temperature for 30 min, then incubated at 4 °C overnight, and stored at −20 °C [39–41]. Two microliters of the Tn5 transposome complex were used for electroporation into SE PT13A as described above. Kanamycin resistance colonies were selected on LB plates supplemented with kanamycin (50 µg/ml) and subsequently collected to form a complex library of EZ-Tn5 mutants. The library was stocked at −80 °C in 30 % glycerol. Electroporation using the transposome complex in both methods was performed in three independent trials.

Illumina Sequencing Sample Preparation

Genomic DNA was extracted using QIAamp DNA Mini kit (Qiagen, Valencia, CA) from SE PT13A EZ:Tn5 mutant library, and the concentration and purity were checked with a NanoDrop 1,000 spectrophotometer (Thermo Scientific). Subsequently, the Tn5-junctions of the extracted genomic DNA were specifically amplified using the following protocol: Tn5-junction sequences were enriched from the genomic DNA of the mutant library using a single primer extension [42] with EZ: Tn5 specific primer for right end (RE) and cloned *pfu* DNA polymerase (Agilent Technologies, La Jolla, CA) resulting in fragments with variable lengths. The PCR reaction was then purified with MinElute PCR purification kit (Qiagen, Valencia, CA) and eluted in 10 µl of EB buffer as directed by the manufacture's guide prior to the addition of deoxycytosine homopolymer tail (C-tail) using Terminal Transferase (TdT) following a previous protocol [43].

After heat inactivation of TdT, the reaction was subsequently used as a template in a two steps exponential PCR reactions using ExTaq DNA polymerase (Takara Bio Inc.). The first exponential PCR reaction was performed with short forward Tn5 primer (IR-W) and C-tail linker primer for 20 cycles. The PCR product from this first reaction step was amplified in a second exponential PCR reaction using ExTaq DNA polymerase (Takara Bio Inc.) with long forward barcoded primer (IR2 + IS) and HTM-primer for 25 cycles to add an adapter containing Illumina sequence at both ends (Table 1). The PCR product in the range of 200–300 bp from last reaction was PAGE-purified, dissolved in ultra pure water. DNA concentration and purity were measured with the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA) using Qubit® Assay Kits (dsDNA BR Assay) following the manufacture's manual. The gel-purified PCR amplicons containing Tn5-junction sequences were sequenced (being spiked into other unrelated samples) using an Illumina MiSeq sequencer for 100 cycles at the Center for Food Safety in the Department of Food Science at the University of Arkansas, Fayetteville, AR.

Table 1 Oligonucleotides used in Tn-seq method in this study

Oligonucleotide	Sequence (5′ → 3′)
EZ: Tn5 primer RE	5-CTAGCCAACAAGAGCTTCAGGGT-3
IR2 primer + IS	5-AATGATACGGCGACCACCGAGATCTAC ACTCTTTCCCTACACGACGCTCTTCCGA TCTNNNNAGXXXXXXTCAGGGTTGAGA TGTGTATAAGGGACAG-3
HTM-primer	5-CAAGCAGAAGACGGCATACGAGCTCT TCCGATCTGGGGGGGGGGGGGGG-3
IR-W	5-TCAGGGTTGAGATGTGTATAAGAG ACAG-3

NNNN random sequence for efficient cluster analysis

XXXXXX 6nt barcode sequence. This barcode was designed to allow sorting of the sequence reads, but this feature was not used in this study

Illumina Sequencing Data Analysis

Custom Pearl script was used to analyze the data. Briefly, the sequence reads of 100 bp from Illumina sequencing were analyzed to find and retain the reads that contain 34 bp region corresponding to the Tn5 sequences. Then, 60 bp region corresponds to Tn5-flanking region was extracted and used in the next step. A local blast database of the complete SE P125109 genome (accession no. NC_011294) [44] was created and used for genome mapping of 60 bp of the Tn5-junction sequences. The final output data obtained by this script contained the information on the Tn5-junction sequence and genomic coordinate corresponding to EZ: Tn5 insertion site. The output data were processed using JMP Pro 11 (SAS, Cary, NC). The length of the transposon-junction sequences extracted by this method is long enough for unambiguous identification of the genomic locations from which the insertions were originated.

Statistical Analysis

Each method was carried out using the transposome complex three times independently. The comparison of improved mutagenesis efficiency was analyzed using JMP Pro 11 statistical software (SAS Institute, Cary, NC). The Student's t test for significance was used, with the statistical significance set at $P < 0.05$.

Results

Our goal was to form a comprehensive saturated mutant library of SE as a stock to be used in various functional genomics analyses. Initially, we applied the heat-inactivation method by Edward et al. [36] for temporarily inactivating SE restriction-modification systems. As a result, the mutagenesis efficiency exhibited an increase of only about 12-fold which is statistically significant ($P < 0.05$) (Table 2).

Since the increase of mutagenesis was not adequate to achieve a saturated complex library, we examined other methods by which we could improve the efficiency. We chose the method by Colegio et al. [37] using adapted DNA to improve transposon mutagenesis of SE. The result presented in Table 3 clearly demonstrates the increase in the number of transposon mutants per electroporation. There was an approximately 46-fold increase in the efficiency of transposon mutagenesis compared to non-adapted transposon DNA, resulting in an average of 12,626 colonies per electroporation reaction with a transformation efficiency of 1.58×10^5cfu/μg after the addition of pre-warmed SOC medium. This increase is significantly different when comparing the results from Table 3 and also when comparing the results with Table 2.

Table 2 Effect of heat-inactivation step on the efficiency of Tn5 mutagenesis in *S.* Enteritidis

Condition	Average cfu/μg DNA	Standard error
No heat	4.65E+03	±9.60E+02
Heat	5.62E+04	±3.95E+03

This experiment was performed in three independent trials

Table 3 Effect of host adaptation on the efficiency of Tn5 mutagenesis in *S.* Enteritidis

Condition	Average cfu/μg DNA	Standard error
No adaptation	3.38E+03	±1.88E+02
Host adaptation	1.58E+05	±4.13E+03

This experiment was performed in three independent trials

The data obtained using illumina sequencing after analysis revealed that there were 95,780 reads containing Tn5 sequences, among which 30,594 reads were mapped perfectly on SE genome (accession no. NC_011294). From the Illumina sequencing reads result, there were 30,594 unique insertions located on the genome (100 %). These transposon insertion sites were confirmed using nucleotide local BLAST program and exhibited a random distribution pattern throughout the genome of SE with the global view through JMP Pro 11 system (SAS, Cary, NC) (Fig. 1). Although the distribution pattern showed coverage throughout the genome, there is still one large gap of approximately 37,103 bp falls in between the locations 2,018,270 bp and 2,055,373 bp of SE reference genome. We identified the genes of that region in the reference genome and determined that they mainly belong to phage and phage-related products. This accurately reflects the genomic region uniquely present in the reference genome (phage type 4), but absent in SE strain (phage type 13A) used in our study.

Discussion

Numerous strategies have been developed to enhance and/or overcome the problem(s) that negatively influence the transformation efficiency. Even among different serovars of *Salmonella*, some methods work ideally with some bacterial strains, while other methods fail to yield any improvements. Heat was used to increase the DNA transformation efficiency either among different strains (intra-species) or different species (inter-species) by temporarily inactivating the restriction barriers during the preparation of electro-competent cells [36]. Plasmid DNA from *E. coli*

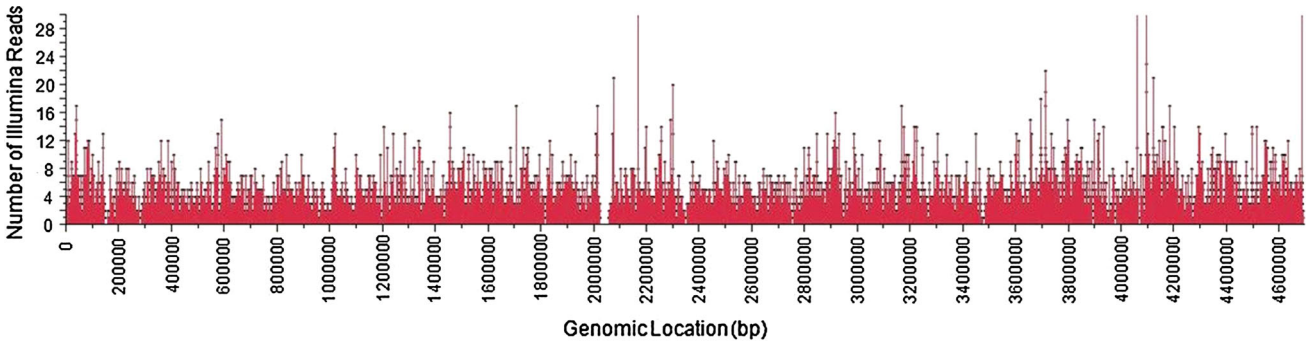

Fig. 1 Global view of Tn5 insertion sites on the *S*. Enteritidis genome

was electro-transformed into heat pre-treated SE competent cells and resulted in enhanced transformation efficiency by recovering about 2,600 colonies [36].

Transposon mutagenesis has broad application in functional analysis of bacterial genes by generating random insertional mutations. For convenient and efficient transposon mutagenesis in diverse bacterial species, an in vivo transposon mutagenesis was developed by Goryshin et. al [39]. In this system, a pre-formed transposon–transposase complex (transposome complex) is electro-transformed into the host bacterial cells resulting in high and efficient transposition frequency. We used this method in attempt to construct a comprehensive library of random transposon insertions, but were faced by the obstacle of host restriction system that significantly reduced the number of random mutants. As a result, we examined other techniques in combination with the in vivo transposon mutagenesis to improve the efficiency of random transposon mutagenesis.

The result from Table 2 suggests that inactivation of the host restriction system by heat treatment was only moderately effective for transposon mutagenesis. By recovering a host-modified vector from SE, we were able to construct a transposome complex that can be used for transposon mutagenesis with significantly increased transformation efficiency (Table 3). Most genetic screening studies with *Salmonella* serotypes have been performed with ST partially due to the limitations associated with other serotypes in performing genetic studies. A study by Shah and others identified virulence genes essential for chicken infection using a small library of the serotype Gallinarum constructed through PCR and signature-tagged mutagenesis [45]. A Tn-seq method was developed previously in our lab with a modified EZ: Tn5 transposon and successfully used to identify genetic determinants of ST that are conditionally essential for growth or survival under various conditions [15]. The disadvantage of this version was the short sequence of Tn5-junctions that result in ambiguous mapping of large portion of the reads to multiple genomic locations on the genome. The method described in this study to amplify Tn5-junctions overcame the limitation with a long Tn5-junction sequence that can be mapped solely to single locations of genomic DNA.

Although SE has been a major public health issue for the last three decades, not many genomes of this serovar strains have been sequenced. A recent publication by Timme reported the draft of genome sequenced for 21 strains of SE [46]. *Salmonella* Enteritidis strain P125109 [44] and, more recently, LA5 strain [47] are the only strains with completed genome sequences, but more genome completed sequences will support in-depth genetic analysis of SE and potentially the identification of more host-specific restriction-modification systems in *Salmonella* that are unique to the species or even strains-specific systems [48–51].

Overcoming the restriction system barriers of bacteria has been a major obstacle for various routine genetic manipulations in bacteria. It is not clear yet the reasons that some serotypes of *Salmonella* are less efficient in transformation than other serotypes. Other alternative approaches or combinations of those may help improve the transformation efficiency and transposon mutagenesis. We believe that the simple strategy tested in this study using host-adapted EZ: Tn5 transposon can be applied to other species to create more comprehensive Tn5 insertion libraries and facilitate functional genomics studies.

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