Analysis of *Escherichia coli* K1 virulence genes by transposon-directed sequencing

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Abstract Transposon-directed insertion site sequencing (TraDIS) combines random transposon mutagenesis and massively parallel sequencing to shed light on bacterial gene function on a genome-wide scale and in a high-throughput manner. The technique has proven successful in the determination of the fitness contribution of every gene under specific conditions both *in vitro* and *in vivo*. In this contribution we describe the procedure used for the identification of *Escherichia coli* K1 genes essential for *in vitro* growth, survival in pooled human serum and gastrointestinal colonization in a rodent model of neonatal invasive infection. TraDIS has broad application for systems-level analysis of a wide range of pathogenic, commensular and saprophytic bacteria.

Key words transposon-directed insertion site sequencing, essential genes, *Escherichia coli*, systems microbiology, bacterial pathogenesis, bacterial fitness

1. Introduction

The development of massively parallel sequencing in combination with more established transposon mutagenesis techniques has enabled genome-wide enhanced genetic screening of large mutant pools, and for the first time provides a high-throughput means of assigning function to multiply disrupted genes in a single experiment. A number of variations of transposon sequencing were described simultaneously and independently in 2009, and variously referred to as transposon insertion site sequencing (Tn-seq) [1], insertion sequencing (INSeq) [2], high-throughput insertion tracking by deep sequencing (HITS) [3] and transposon-directed insertion site sequencing (TraDIS; Fig.1) [4]. All rely on the creation of a pool of insertion mutants in which every gene has been disrupted at multiple sites followed by determination of the sites of transposon insertion through sequencing of transposon junctions within chromosomal DNA before (input pool) and after (output pool) the application of a specific condition. The large majority of studies have employed either random mutagenesis using transposon Tn*5* or mariner transposon derivatives that target thymine-adenine dinucleotides [5]. Mutants carrying genes essential or required under a specific condition will be outcompeted by other mutants and will be underrepresented in the output pool. Although initially used to examine the relationship between different *in vitro* growth conditions and gene essentiality on a genome-wide scale, transposon sequencing has provided insights into the role of key genes required for fitness of a wide range of bacterial pathogens during various stages of the infection process in laboratory animal models [5-7].

We have a longstanding interest in the capacity of *Escherichia coli* strains expressing the α-2,8-linked polysialic acid capsular K1 polysaccharide to cause potentially lethal sepsis and meningitis in new born children and to function as a benign but frequent component of the gut microbiota in non-neonatal infants and adults [8-10]. These bacteria are acquired by the neonate at birth and colonize the small intestine prior to invasion of the blood compartment and central nervous system. In order to identify genes essential for gastrointestinal colonization and survival in blood or serum,

prerequisites for experimental invasive infection in neonatal rat pups, we employed a TraDIS library and constituent sub-libraries of ~775,000 Tn*5* transposon mutants of the highly virulent *E. coli* K1 strain A192PP [11]. The processing of bacterial DNA is illustrated in Fig. 2. This study enabled the identification of a number of bacterial genes that had not previously been implicated in invasive *E. coli* K1 disease but also highlighted one of a number of confounding factors that must be borne in mind during analyses of gene essentiality [12]: the presence of a bottleneck during bacterial translocation from gut to blood and from the blood circulation to cerebrospinal compartment invalidated TraDIS analysis at these physical barriers by restricting bacterial population diversity, thereby eliminating genotypes from the translocated gene pool in a stochastic, rather than gene-dependent, manner [11]. Here we describe the generation of a TraDIS library and its use as a tool to determine gene essentiality for *E. coli* K1 pathogenesis; the procedures are sufficiently robust for analytical use to determine gene function for a broad range of bacteria in a wide variety of scenarios.

2. Materials

Unless otherwise stated, we use ultrapure water when referring to water. All buffers described below are filter-sterilized (≤ 0.45 μm) after preparation.

2.1 Preparation of electrocompetent *E. coli*

1. *E. coli* strain cultured on an appropriate agar plate.
2. Sterile loop.
3. Sterile culture vessels.
4. Luria-Bertani (LB) broth.
5. Optical density meter for tubes.
6. 10% glycerol: Mix 100 mL glycerol with 900 mL dH_20.
7. Sterile 1.5 mL microcentrifuge tubes.

2.2 Transformation of *E. coli* with EZ-Tn*5* tranposome

1. Electrocompetent *E. coli*.
2. 2 mM electroporation cuvettes.
3. MicroPulser Electroporator or equivalent.
4. Sterile 1.5 mL microcentrifuge tube.
5. EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (Epicentre)
6. SOC medium: 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose*. Add glucose to autoclaved and cooled solution containing the remaining ingredients. Filter sterilise the final solution with a 0.2 μM filter.
7. Mueller-Hinton agar plates supplemented with 50 μg/mL kanamycin.
8. Sterile spreader.

2.3 Harvest and pool *E. coli* Tn*5* mutants

1. Sterile 15 mL centrifuge tube.

2. 20% glycerol in PBS: Mix 200 mL glycerol with 800 mL PBS.
3. Cell scrapers.

2.4 Count colony-forming units in *E. coli* Tn*5* pool

1. PBS (1X)
2. Mueller-Hinton agar plates supplemented with 50 µg/mL kanamycin.

2.5 Extract DNA from *E. coli* Tn5 library

1. DNA extraction kit (E.g. PureElute Bacterial Genomic DNA preparation kit; EdgeBiosystems, UK)
2. Sterile dH_20.
3. PCR purification kit (E.g. QIAgen MinElute PCR purification kit).
4. 1.2% agarose gel, containing ethidium bromide or alternative DNA dye.
5. NanoDrop SpectroPhotometer.

2.6 Fragmentation of DNA

1. Sterile dH_20.
2. Sonication device (E.g. Biorupton Sonication Device).
3. PCR purification kit (E.g. QIAgen MinElute PCR purification kit).
4. 1.2% agarose gel containing DNA visualisation dye (E.g. Ethidium bromide).
5. TBE buffer (1X): 89 mM Tris-borate, 2mM EDTA at pH 8.3.
6. DNA ladder (E.g. 100 bp DNA ladder, NEB).
7. DNA gel loading dye (6X).
8. Imaging System (E.g. ChemiDoc imaging system).

2.7 Repairing the ends of fragmented DNA

1. Sterile dH_20.
2. NEBNext DNA library prep reagent kit for Illumina.

3. PCR purification kit (E.g. QIAgen MinElute PCR purification kit).

2.8 Addition of A-tail to repaired DNA fragments

1. Sterile dH_20.
2. NEBNext DNA library prep reagent kit for Illumina.
3. PCR purification kit (E.g. QIAgen MinElute PCR purification kit).
4. Agilent High Sensitivity DNA analysis kit and Bioanalyzer (Agilent)

2.9 Ligation of adapters to DNA fragments

1. Sterile dH_20.
2. NEBNext DNA library prep reagent kit for Illumina.
3. PCR purification kit (E.g. QIAgen MinElute PCR purification kit).
4. Agilent 1000 DNA kit and Bioanalyzer (Agilent)
5. Adapter Ind_Ad_T at 100 mM (desalted, and with phosphorothioate): diluted in dH_20.

   ACACTCTTTCCCTACACGACGCTCTTCCGATC*T
6. Adapter Ind_Ad_B at 100 mM (desalted): diluted in dH_20.

   pGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC

2.10 PCR amplification of Tn insertion sites

1. HotStarTaq polymerase mix.
2. Adapt FO at 100 mM (desalted): diluted in dH_20.

   CGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGGGGATCCTCTAGAGTCGACCTGC

   Adapt RO at 100 mM (desalted): diluted in dH_20.

   GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACACTCTTTCCCTACACGACGCTCTTCCGATC
3. Sterile dH_20.

4. Sterile PCR tubes.

2.11 Purification of PCR amplicons

1. UV transilluminator box.
2. 1% agarose gel containing DNA visualisation dye (E.g. Ethidium bromide).
3. TBE buffer (1X): 89 mM Tris-borate, 2mM EDTA at pH 8.3.
4. DNA ladder (E.g. 100 bp DNA ladder, NEB).
5. DNA gel loading dye (6X): Dissolve 25 mg bromophenol blue, 25 mg xylene cyanol FF and 1.5 g Ficoll 400 in 10 mL Milli-Q dH_20.
6. DNA gel extraction kit (E.g. QIAquick Gel purification kit)
7. Agilent High Sensitivity DNA analysis kit and Bioanalyzer (Agilent)

2.12 Sample indexing

1. Kapa HotStart ReadyMix (2X) (Kapa Biosystems).
2. Nextera XT 24 indexes (Illumina).
3. Sterile PCR tubes.

2.13 Clean-up size indexed fragments

1. AMPure XP beads (Beckman Coulter).
2. Sterile 96-well plate.
3. 96-well plate seal.
4. 96-well plate shaker.
5. 100% ethanol.
6. Sterile dH_20.

7. Tris buffer: 10 mM Tris in water, pH8.5.

8. Agilent High Sensitivity DNA analysis kit and Bioanalyzer (Agilent).

2.14 Sequencing on MiSeq

1. MiSeq Reagent Kit v2 (300-cycle)

3. Methods

3.1 Preparation of electrocompetent *E. coli*

1. Pick a single colony of *E. coli* strain from an appropriate agar plate using a sterile loop.
2. Inoculate 10 mL of Luria-Bertani (LB) broth in a sterile culture vessel.
3. Incubate at overnight at 37^{o}C with shaking.
4. Add 0.5 mL of the *E. coli* culture to 50 ml of LB broth in a sterile culture vessel.
5. Incubate the *E. coli* culture at 37^{o}C
6. At regular intervals measure the absorbance at OD_{600}.
7. When absorbance at OD_{600} = 0.4, place the *E. coli* culture on ice for 20 minutes (*see* **Note 1**).
8. Centrifuge the *E. coli* culture for 15 minutes at 4^{o}C and 4000 x g.
9. Remove supernatant.
10. Re-suspend the *E. coli* pellet in 25 mL of ice-cold sterile 10% glycerol.
11. Centrifuge the *E. coli* culture for 15 minutes at 4^{o}C and 4000 x g.
12. Remove supernatant.
13. Re-suspend the *E. coli* pellet in 10 mL of ice-cold sterile 10% glycerol.
14. Centrifuge the *E. coli* culture for 15 minutes at 4^{o}C and 4000 x g.
15. Remove supernatant.
16. Re-suspend the *E. coli* pellet in 1 mL of ice-cold sterile 10% glycerol.
17. Aliquot 200 µl of the electrocompetent *E. coli* into 5 x sterile 1.5 mL microcentrifuge tubes (*see* **Note 2**).
18. Use immediately or store aliquots at -80^{o}C.

3.2 Transformation of *E. coli* with EZ-Tn*5* transposome

1. Place 1 vial of electrocompetent *E. coli* on ice for 15 minutes (*see* **Note 3**).

2. Place 2 mM electroporation cuvette and 1.5 mL microcentrifuge tube on ice for 15 minutes (*see* **Note 4**).
3. Pipette 60 µl of electrocompetent *E. coli* cells into a sterile 1.5 mL microcentrifuge tube, and immediately place on ice.
4. Add 0.2µl of EZ-Tn*5* transposome to the electrocompetent *E. coli* cells.
5. Mix by gently pipetting up and down.
6. Transfer the mixture to a 2mM electroporation cuvette.
7. Place electroporation cuvette into a MicroPulser Electroporator or equivalent, and run electroporation using Ec2 (2.5Kv, 25µF, 200Ω) settings.
8. Add 1 mL of SOC medium to the electroporation cuvette.
9. Mix by gently pipetting up and down.
10. Transfer the *E. coli* cells to a sterile 1.5 mL microcentrifuge tube.
11. Incubate at 37^{o}C for 60 minutes.
12. Pipette 100 µL of *E. coli* culture onto Mueller-Hinton agar plates supplemented with 50 µg/mL kanamycin.
13. Spread the culture across the plate using a sterile spreader.
14. Repeat steps 11 and 12 until all *E. coli* culture is spread on a plate.
15. Incubate the plates at 37^{o}C overnight.

3.3 Harvest and pool *E. coli* Tn*5* mutants

1. Pipette 3 mL of sterile 20% glycerol in PBS to a 15 mL centrifuge tube.
2. Count the number of kanamycin resistant *E. coli* colonies on a plate.
3. Using a cell scraper, collect all *E. coli* colonies from a plate and re-suspend in the 20% glycerol in PBS. Use a vortex to assist the re-suspension of colonies in 20% glycerol in PBS.
4. Repeat steps 2 and 3, until 3000-5000 *E. coli* colonies are pooled into one tube.
5. Note the number of colonies in the mini-pool, and store at -80^{o}C until required.

6. To form a large *E. coli* Tn*5* pool, defrost mini-pools of *E. coli* Tn*5* on ice and mix 100 μL of each mini-pool in a 15 mL centrifuge tube.

3.4 Count colony-forming units in *E. coli* Tn*5* pool

1. Defrost pool of *E. coli* Tn*5* on ice.
2. Mix 20 μL of *E. coli* Tn*5* pool with 180 μL of sterile PBS.
3. Repeat step 2 for 10 serial dilutions.
4. Pipette 50 μL of each serial dilution onto Mueller-Hinton agar plates supplemented with 50 μg/mL kanamycin.
5. Incubate the plates at 37°C overnight.
6. Count colony-forming units (CFU) per mL.

3.5 Extract DNA from *E. coli* Tn*5* library

1. Extract DNA from an aliquot of the *E. coli* Tn5 library using a selection of standard DNA extraction kits.
2. Elute DNA into sterile dH_20.
3. Measure the concentration of purified DNA using NanoDrop SpectroPhotometer.

3.6 Fragmentation of DNA

1. Dilute DNA to 10 μg/mL in sterile dH_20, to a total volume of 200 μL.
2. Transfer DNA sample to sterile 1.5 mL microcentrifuge tube.
3. Load microcentrifuge tube into sonication device.
4. Add ice and water to the sonication device vessel to cover the microcentrifuge tubes but not the microcentrifuge tube holder.
5. Sonicate on medium density for 15 minutes, set at 30 seconds on / 30 seconds off.
6. Repeat steps 4 and 5.
7. Purify fragmented DNA using a selection of standard PCR purification kits.

8. Elute the purified fragmented DNA into 80 µl of dH_20.
9. Load a 1.2% agarose gel containing a DNA visualisation dye into a gel electrophoresis tank, and cover with TBE buffer.
10. Add 6 µL of an appropriate DNA ladder into the first well of the agarose gel.
11. Mix 5 µL of purified fragmented DNA with 1 µL of DNA gel loading dye.
12. Load the sample into the well of a 1.2% agarose gel.
13. Run the gel at 110 V for 30 minutes.
14. Image the gel using imaging system.
15. If fragmentation has been successful, the DNA should appear as a smear with a mean band size of around 500 bp.

3.7 Repairing the ends of fragmented DNA

1. To repair DNA ends, use the NEBNext DNA library prep reagent kit for Illumina.
2. For each 75 µL sample of purified fragmented DNA, add:-

    10.00 µL phosphorylation buffer

    4.00 µL dNTP mix

    1.50 µL Klenlow DNA polymerase

    5.00 µL T4 PNK

    5.00 µL T4 DNA polymerase
3. Gently pipette up and down to mix reagents.
4. Incubate the microcentrifuge tubes at room temperature for 30 minutes.
5. Purify DNA fragments using a selection of standard PCR purification kits.
6. Elute the purified fragmented DNA into 35 µL of sterile dH_20.

3.8 Addition of A-tail to repaired DNA fragments

1. To polyadenylate the ends of repaired DNA, use the NEBNext DNA library prep reagent kit for Illumina.
2. For each 35 µL sample of purified repaired DNA fragments, add:-

    5.00 µL NEB2 buffer

    10.00 µL dATP

    3.00 µL Klenlow fragment (exo-)
3. Gently pipette up and down to mix reagents.
4. Incubate the microcentrifuge tube at 37 ^{o}C for 1 hour (best performed in water bath)
5. Purify DNA fragments using a selection of standard PCR purification kits.
6. Elute the purified fragmented DNA into 22 µL of sterile dH_20.
7. Measure concentration of DNA sample and mean fragment size using Agilent 1000 DNA kit and BioAnalyzer following standard protocol.

3.9 Ligation of adapters to DNA fragments

1. For each sample, mix the following reagents in a microcentrifuge tube:-

    0.40 µL Adapter Ind_Ad_T

    0.40 µL Adapter Ind_Ad_T

    0.40 µL 10X annealing buffer

    2.80 µL dH20
2. Gently pipette up and down to mix reagents.
3. Incubate the microcentrifuge tube as follows in a PCR machine:-

    98^{o}C for 2 minutes

    70^{o}C for 5 minutes

    55^{o}C for 5 minutes
4. Incubate the microcentrifuge tube on ice for 15 minutes.
5. Calculate the amount of adapters required per sample using the following equation:-

(Adaptor excess x DNA concentration x sample DNA volume x 25)/650 x mean DNA size

= (10 x ? x 17.5 µl x 25)/650 x ?

6. Calculate the volume of 2x buffer required per sample using the following equation:-

   (sample DNA volume + adapter volume + ligase volume)

   = (17.5 µl + ? + 5 µl)

7. For each sample, mix the following in a sterile 1.5 ml microcentrifuge tube:-

   17.50 µL fragmented DNA sample

   ? µL annealed adapters (volume calculated in step 4)

   ? µL 2x ligation buffer (NEB kit; volume calculated in step 5)

   5.00 µL ligase

8. Gently pipette up and down to mix reagents.
9. Incubate microcentrifuge tubes at room temperature for 15 minutes.
10. Purify DNA fragments using a selection of standard PCR purification kits.
11. Elute the purified fragmented DNA into 35 µL of sterile dH_20.

3.10 PCR amplification of Tn insertion sites

1. In order to minimise amplification bias, PCR is best performed on small volumes of the ligated material in 10 parallel reactions.
2. Mix a PCR mastermix in a microcentrifuge tube as follows:-

   150.0 µL of HotStarTaq polymerase mix

   3.60 µL of F primer (Adapt RO at concentration 100 µM)

   3.60 µL of R primer (Tn FO at concentration 100 µM)

   118.80 µL sterile dH_2O

3. Gently pipette up and down to mix reagents.
4. Aliquot 24.00 µL of reaction mix into 11 sterile PCR tubes.

5. Add 1.00 µL of the sample to each of 10 PCR tubes.
6. Add 1.00 µL of sterile dH_2O to control PCR tube.
7. Run the PCR on the following program:-

    94^oC 15 mins

    94^oC 45secs)

    60^oC 45secs) x22 cycles

    72^oC 45secs)

    72^oC 10mins

    10^oC continuous

3.11 Purification of PCR amplicons

1. Prepare a deep 1% agarose gel using a wide well comb containing DNA visualisation dye (*see* **Note 1**)
2. Load the 1% agarose gel containing a DNA visualisation dye into a gel electrophoresis tank, and cover with 1 X TBE buffer.
3. Add 6 µL of an appropriate DNA ladder into the first well of the agarose gel.
4. Mix each 25 µL of PCR product with 5 µL of DNA gel loading Dye 6X.
5. Load as much sample into the wells of a 1% agarose gel (*see* **Note 1**).
6. Repeat step 5 until all PCR products are loaded into wells.
7. Run the gel at 70 V for 90 minutes.
8. Image the gel on a UV transilluminator.
9. Using a sterile scalpel blade, cut out the band of gel from each lane that comprises size 150 to 700 bp.
10. Purify DNA fragments using a selection of standard PCR gel extraction purification kits.
11. Elute the purified fragmented DNA into 25 µL of sterile dH_20.

12. Measure concentration of DNA sample and mean fragment size using Agilent 1000 DNA kit and BioAnalyzer following standard protocol.

3.12 Sample indexing

1. In a sterile PCR tube, mix the following:-

    5.00 µL of DNA sample

    5.00 µL of Nextera index primer 1 (N7XX)

    5.00 µL of Nextera index primer 2 (S5XX)

    25.00 µL of 2 x Kapa Hifi HotStart Ready Mix

    10.00 µL of sterile dH_20

2. Gently mix by pipetting up and down.
3. Incubate the PCR tube as follows:-

    1 cycle: 95°C for 3 minutes

    8 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds

    1 cycle: 72°C for 5 minutes

4. Store samples on ice.

3.13 Clean-up size indexed fragments

1. Place AMPure XP beads at room temperature for 30 mins.
2. Shake the AMPure XP beads for 1 minute.
3. Add 56 µL of AMPure XP bead mixture to a well of sterile 96-well plate.
4. Add 50 µL of indexing PCR product.
5. Gently pipette up and down 10 times to mix the sample with AMPure XP beads.
6. Incubate the 96-well plate at room temperature for 5 minutes.
7. Cover the plate with a 96-well plate seal.
8. Place the 96-well plate on a shaker for 2 minutes at 1800 rpm.

9. Incubate the 96-well plate at room temperature for 5 minutes.
10. Place the 96-well plate on a 96-well plate magnetic stand for 2 minutes.
11. Using a pipette, carefully remove supernatant from wells without disturbing the AMPure XP beads on the bottom of the well.
12. Mix 4 mL of 100% ethanol with 1 ml of sterile dH_20 in a 15 mL centrifuge tube.
13. Add 200 µL of fresh 80% ethanol to the well.
14. Incubate the 96-well plate at room temperature for 1 minute.
15. Using a pipette, carefully remove supernatant from wells without disturbing the AMPure XP beads on the bottom of the well.
16. Repeat steps 13, 14 and 15.
17. Incubate the 96-well plate at room temperature for 10 minutes.
18. Add 27.5 µL 10 mM Tris pH8.5 to the well.
19. Remove the 96-well plate from the 96-well plate magnetic stand.
20. Gently pipette up and down 10 times to mix the Tris buffer with AMPure XP beads.
21. Incubate the 96-well plate at room temperature for 2 minutes.
22. Place the 96-well plate on the 96-well magnetic stand for 2 minutes.
23. Using a pipette, carefully transfer the supernatant from wells to a sterile 1.5 mL. microcentrifuge tube without disturbing the AMPure XP beads on the bottom of the well.
24. Measure concentration of DNA sample and mean fragment size using Agilent 1000 DNA kit and BioAnalyzer following standard protocol.

3.14 Sequencing of amplicons with MiSeq

1. Perform

3.15 Processing of sequence reads

Notes

1. *E. coli* should be kept chilled at all subsequent stages in order to maximise electro-competency. All reagents and plasticware should be chilled at 4°C or on ice. Centrifugation steps should be performed at 4°C.
2. It is beneficial to place the 1.5 mL microcentrifuge tubes on ice prior to aliquotting.
3. In our experience, there is enhanced transformation of electrocompetent *E. coli* that are freshly prepared compared to those previously stored at -80°C.
4. Electroporation cuvettes can also be stored at -20°C.
5. We find that it is more efficient for down-stream purification to maximise the amount of PCR product loaded into a single well of a 1% agarose gel. Therefore, 1% agarose gels should be prepared that are deep and using a comb that generates wide wells. This permits purification of DNA from a smaller quantity of agarose gel.

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Legends

Fig. 1 Overview of the TraDIS procedure

Fig. 2 Processing of bacterial DNA for TraDIS