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Determination of the optimal chromosomal location(s) for a DNA element in Escherichia coli using a novel transposon mediated approach --Manuscript Draft--

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Abstract:	<p>The optimal chromosomal position(s) of a given DNA element was determined by transposon-mediated random insertion followed by fitness selection. In bacteria, the impact of the genetic context on the function of a genetic element can be difficult to assess. Several mechanisms including topological effects, transcriptional interference from neighboring genes and/or replication-associated gene dosage may affect the function of a given genetic element. Here we describe a method that permits random integration of a DNA element on the chromosome of Escherichia coli and select the most favorable locations using a simple growth competition experiment. The method takes advantage of a well described transposon based system of random insertion, coupled with a selection of the fittest clone(s) by growth advantage, a procedure that is easily adjustable to experimental needs. The nature of the fittest clone(s) can be determined by whole genome sequencing on a complex multi-clonal population or by Easy Gene Walking for a rapid identification of selected clones. Here the non-coding DNA region DARS2 which controls initiation of chromosome replication in E. coli was used as an example. The function of DARS2 is known to be affected by replication-associated gene dosage; the closer DARS2 gets to the origin of DNA replication the more active it becomes. DARS2 was randomly inserted into the chromosome of a DARS2 deleted strain. The resultant clones containing individual insertions were pooled and competed against one another for hundreds of generations. Finally the fittest clones were characterized and found to contain DARS2 inserted in close</p>

	proximity to the original DARS2 location.
Author Comments:	<p>Dear editor</p> <p>We are grateful that you find the manuscript "Determination of the optimal chromosomal location(s) for a DNA element in Escherichia coli using a novel transposon-mediated approach" suitable for Journal of Visualized Experiments. Furthermore, we do appreciate the way the manuscript has been handled by the editor.</p> <p>We have addressed "Protocol Language" comments, "Protocol Detail" comments, "Figure" comments, "References", "Commercial Language" and added Acknowledgments and Disclosures (see below).</p> <p>We hope you find the revised manuscript acceptable for publication in Journal of Visualized Experiments.</p> <p>Kind regards</p> <p>Anders Løbner-Olesen</p>
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Kind regards

A handwritten signature in blue ink, which appears to read 'Anders Løbner-Olesen', is written over a light blue horizontal line.

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MAY 2, 2017

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TITLE:

Determination of the Optimal Chromosomal Location(s) for a DNA Element in *Escherichia coli* Using a Novel Transposon-mediated Approach

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KEYWORDS:

Random transposon insertion pool, culture competition, optimal genomic context, whole-genome sequencing, easy gene walking, cell cycle analysis by flow cytometry

SHORT ABSTRACT:

Here, the power of a transposon-mediated random insertion of a non-coding DNA element was used to resolve its optimal chromosomal position.

LONG ABSTRACT:

The optimal chromosomal position(s) of a given DNA element was/were determined by transposon-mediated random insertion followed by fitness selection. In bacteria, the impact of the genetic context on the function of a genetic element can be difficult to assess. Several mechanisms, including topological effects, transcriptional interference from neighboring genes, and/or replication-associated gene dosage, may affect the function of a given genetic element. Here, we describe a method that permits the random integration of a DNA element into the chromosome of *Escherichia coli* and select the most favorable locations using a simple growth competition experiment. The method takes advantage of a well-described transposon-based system of random insertion, coupled with a selection of the fittest clone(s) by growth advantage, a procedure that is easily adjustable to experimental needs. The nature of the fittest clone(s) can be determined by whole-genome sequencing on a complex multi-clonal population or by easy gene walking for the rapid identification of selected clones. Here, the non-coding DNA region *DARS2*, which controls the initiation of chromosome replication in *E. coli*, was used as an

example. The function of *DARS2* is known to be affected by replication-associated gene dosage; the closer *DARS2* gets to the origin of DNA replication, the more active it becomes. *DARS2* was randomly inserted into the chromosome of a *DARS2*-deleted strain. The resultant clones containing individual insertions were pooled and competed against one another for hundreds of generations. Finally, the fittest clones were characterized and found to contain *DARS2* inserted in close proximity to the original *DARS2* location.

INTRODUCTION:

The function of any genetic element can be affected by its location in the genome. In bacteria, this mainly results from interference by the transcription of neighboring genes, local DNA topology, and/or replication-associated gene dosage. In particular, the processes of DNA replication and segregation are controlled, at least in part, by non-coding chromosomal regions¹, and the proper function of these regions depends on genomic location/context. In *E. coli*, examples are the *dif* site, required for sister chromosome resolution²; KOPS sequences, required for chromosome segregation³; and *datA*, *DARS1*, and *DARS2* regions, required for proper chromosomal replication control (below; ⁴). We present a method allowing for the random relocation, selection, and determination of the optimal genetic context of any given genetic element, exemplified here by the study of the *DARS2* non-coding region.

In *E. coli*, DnaA is the initiator protein responsible for DNA strand opening at the single replication origin, *oriC*, and for the recruitment of the helicase DnaB⁵⁻⁷. DnaA belongs to the AAA⁺ (*i.e.*, ATPases associated with diverse activities) proteins and can bind both ATP and ADP with similar high affinities⁵. The level of DnaA^{ATP} peaks at initiation⁸, where DnaA^{ATP} forms a multimer on *oriC* that triggers DNA duplex opening⁹. After initiation, *oriC* is made temporarily unavailable for re-initiation due to sequestration by a mechanism involving the binding of the SeqA protein to hemimethylated *oriC*^{10,11}. During sequestration, the level of DnaA^{ATP} is reduced by at least two mechanisms: the regulatory inactivation of DnaA (RIDA)^{12,13} and *datA*-dependent DnaA^{ATP} hydrolysis (DDAH)^{14,15}. Both RIDA and DDAH promote the conversion of DnaA^{ATP} to DnaA^{ADP}. Prior to a new round of initiation, DnaA^{ADP} is re-activated to DnaA^{ATP} at specific DnaA-reactivating sequences (*DARS*): *DARS1* and *DARS2*^{16,17}. The chromosomal *datA*, *DARS1*, and *DARS2* regions are non-coding and act in a chaperone-like manner to modulate DnaA^{ATP}/DnaA^{ADP} interconversion. These regions, located outside the origin of replication, enable the assembly of a DnaA complex for either the inactivation (*datA*;¹⁴) or activation (*DARS1* and *DARS2*;¹⁷) of DnaA. Deleting *DARS2* in a cell does not alter mass doubling time but results in asynchronous replication initiation^{15,16,18}. However, *DARS2*-deficient cells have a fitness cost compared to an otherwise isogenic wildtype during both continuous growth competition in rich medium or during the establishment of colonization in the mouse intestine¹⁸. This indicates that even minor changes in asynchrony/origin concentration have a negative effect on bacterial fitness. In *E. coli*, there is a selective pressure to maintain chromosome symmetry (*i.e.*, two nearly equal length replication arms)¹⁹. The *datA*, *DARS1*, and *DARS2* regions have the same relative distance to *oriC* in all *E. coli* strains sequenced¹⁸, despite large variations in chromosome size.

Here, we use the *DARS2* region of *E. coli* as an example for the identification of the chromosomal position(s) optimal for its function. *DARS2* was inserted into the NKBOR transposon, and the

resultant NKBOR::*DARS2* transposon subsequently inserted randomly into the genome of MG1655 Δ *DARS2*. We thus generated a collection of cells, each possessing *DARS2* placed at a different location on the chromosome. An *in vitro* competition experiment, where all cells in the collection were pooled and competed against each other during continuous growth in LB for an estimated 700 generations, was performed. The outcome of the competition experiment was monitored/determined using Southern blot, easy gene walking, and whole-genome sequencing (WGS; **Figure 1**). End-point clones resolved by easy gene walking were characterized by flow cytometry to evaluate cell-cycle parameters. In a flow cytometric analysis, cell size, DNA content, and initiation synchrony can be measured for a large number of cells. During flow cytometry, a flow of single cells passes a light beam of the appropriate wavelength to excite the stained DNA, which is then simultaneously registered by photomultipliers that collect the emitted fluorescence, a measure of DNA content, provided the cells are stained for DNA. The forward-scattered light is a measure of cell mass²⁰.

The *in vitro* competition experiment we present here is used to address questions relating to the importance of the chromosomal position and genomic context of the genetic element. The method is unbiased and easy to use.

PROTOCOL:

1. Collection of the Transposon Library

Note: The chromosomal *DARS2* locus was cloned into the mini Tn10-based transposon, NKBOR (on pNKBOR)²¹, resulting in NKBOR::*DARS2* (pJFM1). pNKBOR can be obtained online²². pNKBOR is a R6K-based suicide vector that requires the initiator protein π for replication²³. Plasmid pJFM1 is therefore able to replicate in an *E. coli* strain (*e.g.*, Dh5 α λ pir) containing a chromosomal copy of the *pir* gene. However, when pJFM1 is transformed into the Pir-deficient wildtype MG1655, pJFM1 cannot replicate, leading to the selection of kanamycin-resistant clones generated by the random insertions of NKBOR::*DARS2* into the bacterial chromosome. For simplicity, these are referred to as *DARS2* insertions. See **Figure 1** for a schematic presentation of the methodology.

1.1. Prepare electrocompetent MG1655 Δ *DARS2* from actively growing cells in Lysogeny broth (LB) grown at 37 °C²⁴.

1.2. Electroporate pJFM1 into electrocompetent MG1655 Δ *DARS2*, according to Gonzales *et al*²⁴.

1.2.1. Add 1 μ g of pJFM1 (in 1 μ L of water) to 40 μ L of electrocompetent *E. coli* MG1655 Δ *DARS2* and transfer this mixture to a pre-chilled, sterile 0.2-cm gap cuvette. Insert the cuvette into the electroporation chamber. Electroporate at 18 kV, 500 Ω , and 25 μ F; the time constant should be \sim 5.0 ms, and no arcing should occur.

1.2.2. Quickly recover the bacterial suspension by resuspending in 1 mL of pre-warmed LB broth and transfer to a 1.5-mL test tube.

1.2.3. Let the cells recover by incubating under aerated growth conditions at 37 °C for 30 min, without antibiotic selection. Plate the bacteria onto LB agar plates supplemented with 50 µg/mL kanamycin and incubate at 37 °C overnight.

1.3. Count the colonies from the electroporation: one colony is considered equal to one chromosomal transposon insertion.

Note: Colonies can be counted by eye or with a colony counter. The number of colonies is inversely proportional to the average distance separating the transposons located in the chromosome of each clone.

1.4. Add 1 mL of LB broth to each plate and wash off all colonies; 1 mL of LB broth should be sufficient to wash off ~100,000 colonies. Re-use the same 1 mL of LB broth to increase the bacterial concentration. Pool all colonies in the same 50-mL tube.

1.5. Vortex the tube and freeze the start material ($t = 0$). To freeze it, mix 1 mL of cell suspension with 1 mL of 50% glycerol on ice. Transfer the tubes to dry ice for 10 min. Once the cultures are frozen, transfer them to a -80 °C freezer.

Note: A cell concentration of a minimum of 50,000 CFU/mL is expected at this step.

2. Competition Experiment in LB

2.1. Thaw the transposon library from -80 °C on ice. Mix by pipetting.

2.2. Transfer 100 µL of the transposon library to 10 mL of LB in a 15-mL test tube.

2.3. Grow the cells, aerated by continuous shaking (250 rpm), for 8 h at 37 °C to stationary phase (*i.e.*, $OD_{600} = \sim 4.0$). Adjust these parameters to different growth conditions, as desired.

2.4. Propagate the bacterial population by continuous transfers into fresh prewarmed medium every ~10 generations. Do this by transferring 10 µL of the previous stationary phase culture to 10 mL of fresh LB and growing for another 8 h to the stationary phase (*i.e.*, $OD_{600} = \sim 4.0$).

Note: As the start and end optical density are the same, a 1,000-fold dilution corresponds to ~10 generations of growth ($2^{10} = 1,024$). The bacterial population can either be propagated directly into fresh prewarmed medium or saved at 0-4 °C (on ice) and propagated the following day. LB was used here to ensure as many cellular doublings in as short a time as possible (a doubling time close to 22 min).

2.5. After each 100 generations of competition, save five 1-mL samples at -80 °C (as described in step 3.3).

2.6. If the fitness differences between cells containing individual transposon insertions are

expected to be small, keep the duration of competition long; here, 700 generations ($t = 700$) were used.

3. Southern Blot Analysis to Monitor the Competition Experiment Over Time

3.1. Prepare the probe for the Southern blot (1-kb section of the NKBOR) by performing polymerase chain reaction (PCR) amplification using specific primers: NKBOR_Probe_FW: gatgttgacgagtcggaat and NKBOR_Probe_RV: cgttacatccctggcttgtt.

3.1.1. Incubate at 98 °C for 30 s for initial denaturation. Then, perform 35 cycles at 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 45 s. For the final extension, use 72 °C for 10 min.

Note: The template for the PCR reaction was a purified pNKBOR plasmid²¹.

3.1.2. Label the resultant PCR fragment with [α -32P]dATP using the random primer system, according to Smith²⁵.

3.2. Prepare the total cellular DNA according to Løbner-Olesen and von Freiesleben²⁶ for $t = 0$ and selected time points until $t = 700$ estimated generations of competition.

3.3. Digest the total cellular DNA with *PvuI*, which cuts the NKBOR containing the region of interest once only in a region not covered by the probe; 1 unit of *PvuI* can be used to completely digest 1 μ g of substrate DNA.

Note: The probe will recognize fragments containing part of the transposon, along with chromosomal DNA of various lengths, depending on the insertion site.

3.4. Perform a Southern blot using a 0.7% agarose gel according to Løbner-Olesen and von Freiesleben²⁶.

4. Identification of the Fittest Clones

4.1. Use easy gene walking, as described by Harrison *et al.*²⁷, to identify *DARS2* insertion sites from single clones (isolated on LB plates) after 700 estimated generations of competition; the more bands on the Southern blot, the more isolates are needed to cover all transposon insertions.

4.1.1. Isolate genomic DNA for PCR template. Spread bacteria on an LB agar plate containing 50 μ g/mL kanamycin and incubate at 37 °C overnight. Grow single colonies overnight in LB broth at 37 °C and subsequently transfer 20 μ L into 200 μ L of autoclaved distilled water (or use DNA purification, as in step 3.2).

4.1.1.1. Vortex to mix. Heat the mix at 100 °C for 10 min and centrifuge at max speed for 5 min. Save the cell lysate at -20 °C for future use.

4.1.2. Design three nested primers to anneal within the transposon of choice (see **Figure 2** for a graphic representation of the PCR strategy).

Note: Designing Nested Primers should ensure that Nested Primer 3 lies closest to the known 5' end of the transposon DNA, followed by Nested Primers 2 and 1. The spacing between Nested Primer 3 and the 5' end of the known transposon DNA should be sufficient for a sequence read-through from the known DNA to the unknown DNA (to find the specific chromosomal transposon insertion site). For NKBOR the following nested primers were used: pNKBOR_Nested3_RV: gcagggtttattgattcca, pNKBOR_Nested2_RV: tcagcaacaccttcttcacg, and pNKBOR_Nested1_RV: actttctggctggatgatgg. Random primers containing the recognition sites of known restriction enzymes are also used. To ensure a result, one should use at least two different random primers. The restriction sites for the restriction enzymes (*e.g.*, *Sau3AI* and *HindIII*) should be located at the 3' end and preceded by ten random bases so that 5'-NNNNNNNNNNGATC-3' and 5'-NNNNNNNNNNAAGCTT-3' are the primers containing a *Sau3AI* site (GATC) and a *HindIII* site (AAGCTT), respectively, where N = A, T, G, or C.

4.1.3. Use 200 ng of genomic DNA in a 20- μ L PCR reaction. Incubate at 98 °C for 30 s for the initial denaturation. Perform 25 cycles at 98 °C for 30 s, 50 °C for 15 s, and 72 °C for 4 min. For the final extension, use 72 °C for 10 min. Use primer pairs consisting of Nested Primer 1 and one of the random primers.

4.1.4. Use primer pairs consisting of Nested Primer 2 and the same random primer from the second amplification, using 1 μ L of its previous reaction as a template.

4.1.5. Repeat step 4.1.4 with primer pairs consisting of Nested Primer 3 and the same random primer.

4.1.6. Run the products from the final PCR reaction on a 1.5% agarose gel and stain with ethidium bromide. Cut out a band in the range 100-800 bp and isolate the DNA using any commercial DNA gel extracting kit according to the manufacturer's direction. Resuspend the DNA in 15 μ L of water.

Note: Caution. Ethidium bromide is toxic and must be handled with care.

4.1.7. Sequence the band isolated in the previous step (step 4.1.6), with Nested Primer 3 as the sequencing primer, using Sanger sequencing at a commercial provider.

4.2. Perform WGS.

4.2.1. Perform WGS on the total DNA extracted from selected samples collected during the competition experiment, as described by Frimodt-Møller *et al*⁴.

4.3. Perform sequencing analysis.

4.3.1. Align paired-end reads to 150 Ns contiguous to NKBOR, AF310136.1 1,904...2,204 using Bowtie2²⁸ with an interval between seed substrings (S,1,1.15) and a maximum number of ambiguous characters (L,0,0.9).

4.3.2. Select the aligned reads and then re-align to both MG1655 ref|NC_000913.3 and NKBOR gb|AF310136 using blastN²⁹

4.3.3. Assign transposition insertion positions at junctions MG1655-NKBOR.

5. Flow Cytometry

5.1. Collect samples for flow cytometry.

5.1.1. Balance each culture by maintaining it in the exponential growth phase for at least 10 generations. Check the OD₆₀₀ and ensure that it never exceeds 0.3.

5.1.2. Collect two types of flow samples.

5.1.2.1. For RIF-runout, transfer 1 mL of culture to a 15-mL tube containing 30 µL of RIF-CEF (300 µg/mL rifampicin and 36 µg/mL cephalexin dissolved in 12.5 mM NaOH). Leave it at 37 °C for a minimum of 4 h of shaking.

Note: Rifampicin indirectly blocks the initiation of chromosome replication while allowing for ongoing rounds of replication to continue to termination. Cephalexin prevents cell division, which results in replication runout (RIF-runout) and the accumulation of cells with fully replicated chromosomes. The number of fully replicated chromosomes is equal to the number of origins at the time of treatment with rifampicin and cephalexin²⁰.

5.1.2.2. For the EXP-sample, transfer 1 mL of exponentially growing culture to a 1.5-mL tube on ice.

5.2. Fix THE cells as follows. Harvest the cells by centrifugation at 15,000 x g for 5 min at 4 °C. Resuspend the pellet in 100 µL of ice-cold 10 mM Tris pH 7.5 and add 1 mL of ice-cold 77% ethanol. Store the samples at 4 °C until use.

5.3. Stain the cells as follows. Harvest 100-300 µL of fixed cells by centrifugation at 15,000 x g for 15 min. Remove the supernatant and resuspend the pellet in 130 µL of "DNA Staining solution" (90 µg/mL mithramycin, 20 µg/mL ethidium bromide, 10 mM MgCl₂, and 10 mM Tris pH 7.5). Put the samples on ice and in the dark; the samples are ready for flow cytometry analysis after 10 min.

Note: Other DNA stains than ethidium bromide and mithramycin can also be used^{30,31}.

5.4. Determine the origin per cell, the relative cell mass, and the relative DNA content by flow

cytometry analysis.

5.4.1. Perform flow cytometry, as described previously³². Run RIF-runout and EXP-samples using the flow cytometer manufacturer's instructions. For mithramycin- and ethidium bromide-stained cells, use excitation wavelengths 395 and 440 nm and collect the fluorescence above 565 nm.

5.4.2.1. To determine the relative cell mass and relative DNA content, run the EXP-samples to obtain single-parameter forward-light scatter versus cell number histograms and fluorescence intensity versus cell number histograms for a minimum of 30,000 events corresponding to the cell signal.

5.4.2.2. Use forward-scattered light single-parameter distribution to measure the average relative cell mass.

Note: Forward-scattered light is a measure of the average cell mass²⁰.

5.4.2.3. Use fluorescence intensity single-parameter distributions to measure the average DNA content²⁰.

5.4.2.4. Obtain the DNA concentration as the ratio of average DNA content to the average cell mass²⁰.

5.4.3. Determine the number of origins per cell.

5.4.3.1. Run RIF-runout samples to obtain single-parameter fluorescence intensity versus cell number histograms for a minimum of 30,000 events corresponding to the cell signal.

5.4.3.2. Use fluorescence intensity single-parameter distributions to determine the number of chromosomes in each cell²⁰.

5.5. Calculate the asynchrony index (A_i).

5.5.1. Calculate the A_i , as described by Løbner-Olesen *et al.*³², using the fluorescence intensity single parameter distributions of the RIF-runout samples and the formula $A_i = (f_3 + f_5 + f_6 + f_7) / (f_2 + f_4 + f_8)$, where f_x is the fraction cells with x number of fully replicated chromosomes. Consider initiations asynchronous when $A > 0.1$.

REPRESENTATIVE RESULTS:

A Southern blot was done to verify that *DARS2* was distributed randomly throughout the chromosome in the transposon library ($t = 0$) and that the fittest clones would persist over time. The Southern blot was performed on DNA extracted from the initial transposon pool (at $t = 0$) and every estimated 100 out of 700 generations of competition (**Figure 3**). Here, the total cellular DNA from each time-point was digested with the *PvuI* restriction enzyme, known to cut transposon NKBOR::*DARS2* once only in a region not covered by the probe. The Southern blot

was probed with a radioactively labeled DNA fragment complementary to part of NKBOR. As seen from **Figure 3**, the initial *DARS2* pool ($t = 0$) lacked distinct bands, which shows that *DARS2* was inserted randomly throughout the chromosome. Over time, a pattern emerged where the initial large pool of *DARS2* clones developed into only one or a few persisting *DARS2* clones (**Figure 3**; $t = 0$ to $t = 700$).

In the example shown, *DARS2* insertion sites from the competition experiment were identified using WGS and easy gene walking. Here, WGS was used to identify insertion sites from the start pool ($t = 0$) and after 300, 400, and 700 generations of competition. Note that the coverage in the present deep sequencing was insufficient for a complete mapping of insertion sites at $t = 0$; however, it gives a representative subset of the total number of insertions. WGS confirmed the Southern blot result (*i.e.*, the selection of the fittest *DARS2* clones), ending with approximately 98% of all *DARS2* insertions close to the wildtype *DARS2* chromosomal location (*DARS2* Clone IR and Clone *rppH*), while the remaining 2% were elsewhere on the chromosome (*Figure 4*). This strongly suggests that the wildtype position is optimal for *DARS2* function. At $t = 400$, an insertion was found on the opposite replication arm with an almost identical distance to *oriC* as the wildtype *DARS2* position (**Figure 4**), but this insertion was not recovered after 700 generations. Thus, replication-associated gene dosage cannot be the single determinant for optimal position.

Easy gene walking was used to identify *DARS2* insertions sites in single clones isolated after 700 estimated generations of competition. Here, the two *DARS2* insertion sites mentioned above (*DARS2* Clone IR and Clone *rppH*) were identified. Easy gene walking was only done on 20 clones, and this explains why all *DARS2* insertions sites mapped in WGS were not identified. *DARS2*-deficient cells were previously shown to initiate replication in asynchrony and to have a decrease in origin concentration relative to wildtype cells^{4,16-18}. We therefore used flow cytometry to resolve the synchrony in the initiation of DNA replication and cellular origin content for the two selected strains (*DARS2* Clone IR and Clone *rppH*) which, in both cases, were restored to wildtype levels (**Figure 5**). A representative example of a strain possessing a single copy of *DARS2* located in the terminus is shown (**Figure 5E**). Here, the presence of a *DARS2* element in the terminus does not restore synchrony or the cellular origin content to wildtype levels, while the selected *DARS2* clones IR and *rppH* do.

FIGURE LEGENDS:

Figure 1. Methodology overview. Schematic presentation of the methodology. The chromosomal *DARS2* region is cloned into the mini *Tn10* on pNKBOR, creating pJFM1. pJFM1 is transformed into *E. coli* MG1655 $\Delta DARS2$, which triggers a random insertion of *DARS2* linked to *Tn10* onto the chromosome of *E. coli* MG1655 $\Delta DARS2$. Approximately 70,000 clones, each containing a different chromosomal *DARS2* insertion, were pooled and competed directly against each other in LB broth at 37 °C. The direct competition experiment in LB broth was performed for an estimated 700 generations, where a sample was isolated for each 100 generations of direct competition. The total DNA was extracted from each isolated sample and used for Southern blotting and the identification of *DARS2* insertions by WGS.

Figure 2. Graphic presentation of easy gene walking. This figure illustrates a genomic DNA template of an unknown DNA sequence adjacent to a known sequence with priming sites for the random primer and Nested Primers 1, 2, and 3. The results of the three successive amplifications performed using the three designed nested primers are illustrated below. The final product (from round 3) is sequenced using Nested Primer 3. This figure was adapted from Harrison *et al*²⁷.

Figure 3. Southern blot probed for NKBOR. Southern blot analysis of *DARS2* insertions into a *DARS2*-deficient strain. Genomic DNA extracted from every ~100 generations of direct competition, starting at $t = 0$ and ending at 700 generations, were digested with *PvuI* and gel-fractionated. The blot was hybridized with a NKBOR probe (see the **Protocol**). t indicates the number of generations of competition. This figure was adapted from Frimodt-Møller *et al*⁴. HMW and LMW are high-molecular weight and low-molecular weight DNA, respectively.

Figure 4. Graphic representation of resolved transposon insertions sites in $\Delta DARS2$. The positions of *oriC*, *datA*, *DARS1*, *DARS2*, and *terC* are indicated. *DARS2* insertion sites in $\Delta DARS2$ at $t = 0$ (black bars), $t = 400$ (light blue bars), $t = 500$ (red bars), and $t = 700$ (green bars), resolved by full-genome sequencing. This figure was made using DNAPlotter³³ and was adapted from Frimodt-Møller *et al*⁴.

Figure 5. Representative flow cytometry histograms of transposon sites found by easy gene walking at $t = 700$. Cells were grown in AB minimal medium supplemented with 0.2% glucose, 10 $\mu\text{g/mL}$ thiamine, and 0.5% casamino acids at 37 °C. Wildtype and $\Delta DARS2$ are shown in A and B, respectively. Derivatives of the wildtype strain MG1655 devoid of *DARS2* at the original locus and instead carrying a copy of *DARS2* at the resolved transposon site *rrpH*, IR, and the terminus (*terC*) are shown in C, D, and E, respectively. This figure was adapted from Frimodt-Møller *et al*⁴ to show a *DARS2* location that results in a cell cycle anomaly (*terC*) or that restores the wildtype phenotype (*rrpH*, IR).

DISCUSSION:

The methodology used here takes advantage of state-of-the-art techniques to answer a difficult question regarding the optimal genomic position of a genetic element. The random insertion of the genetic element (mediated by the transposon) enables the fast and easy collection of thousands of clones, which then can be made to compete against each other to select for the optimal position of the investigated genetic element (*i.e.*, the fittest clone).

Here, *DARS2* was inserted into the mini-Tn10-based transposon, NKBOR. The choice of transposon is important for the downstream analysis of insertions sites. Several different transposons have been used in transposon-directed insertion-site sequencing (TraDIS) experiments, such as mini-Tn5Km2³⁴ and the more popular choice, the Himar I *Mariner* transposon^{35,36} (for a recent review of this, see van Opijnen and Camilli³⁶). We aimed for 70,000 initial random insertions of *DARS2*, which gives approximately a *DARS2* insertion every 65 bp in the MG1655 genome. This can easily be adjusted by decreasing or increasing the initial pool of colonies collected.

Here, we opted for continuous transfers in LB batch cultures, but the competition experiment can also be performed in a different medium or under different conditions, including using a chemostat³⁷. Furthermore, the procedure can be modified to accommodate any conditions of choice, including various stresses, such as oxidative, osmotic, or antibiotic stress. To verify the presence of persisting clones over time, one can do at least three things: WGS; transposon sequencing (Tn-Seq); or, as here, a Southern Blot. Finally, easy gene walking can be done on a few clones, with the further advantage that transposon insertion sites are identified in single clones, such that the effect of the specific insertion site can be phenotypically analyzed.

The choice of phenotypic assay to evaluate the outcome of a competition experiment depends on the investigated region. We used flow cytometry, which is a powerful method to measure cell-cycle parameters of bacteria. Here, flow cytometry revealed that the selected chromosomal position(s) of *DARS2*, (*i.e.*, the *DARS2* insertions close to the wildtype *DARS2* position) resulted in the correct regulation of replication initiation (*i.e.*, synchrony and DNA concentration). The optimal chromosomal position(s) for *DARS2* were selected in part due the proper replication-associated gene dosage and in part due to the favorable local genomic environment that is important for *DARS2* function⁴.

Here, a non-coding DNA element was investigated, but this could be expanded to any coding region of choice. A recent study found that the level of gene expression varied ~300-fold, depending on its position on the chromosome, without clear correlation to replication-associated gene dosage³⁸. The approach described here could give important insight into the relationship between the genomic location of a particular gene, its transcriptional activity, and the associated fitness advantage. This could in theory assist with the selection of genomic positions, leading to the stronger expression of a given gene, which in turn could be of interest to engineering new, improved strains for recombinant protein production.

Because *E. coli* contains limited intergenic regions³⁹, transposon insertions will, in most cases, disrupt gene(s). This may occasionally create false positives where the fittest clone(s) are not selected due to the optimal chromosomal position of the genetic element in question, but rather because a gene is disrupted—which, in other ways, provides a fitness advantage during the competition experiment⁴.

This methodology takes advantage of an easy-to-use transposon system, where any region of choice can be integrated at random locations. The designed competition experiment can be modified to include any number of selection forces other than pure growth, as seen here. The setup can also be modified to be purely based on Tn-Seq, which yields a greater sequencing resolution of insertions sites than WGS, used here. This unbiased approach should be used to elucidate exciting new features in thus-far uncharacterized organisms, which might show that common trends exist in the chromosomal organization of Eubacteria.

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DISCLOSURES:

The authors have no competing financial interest.

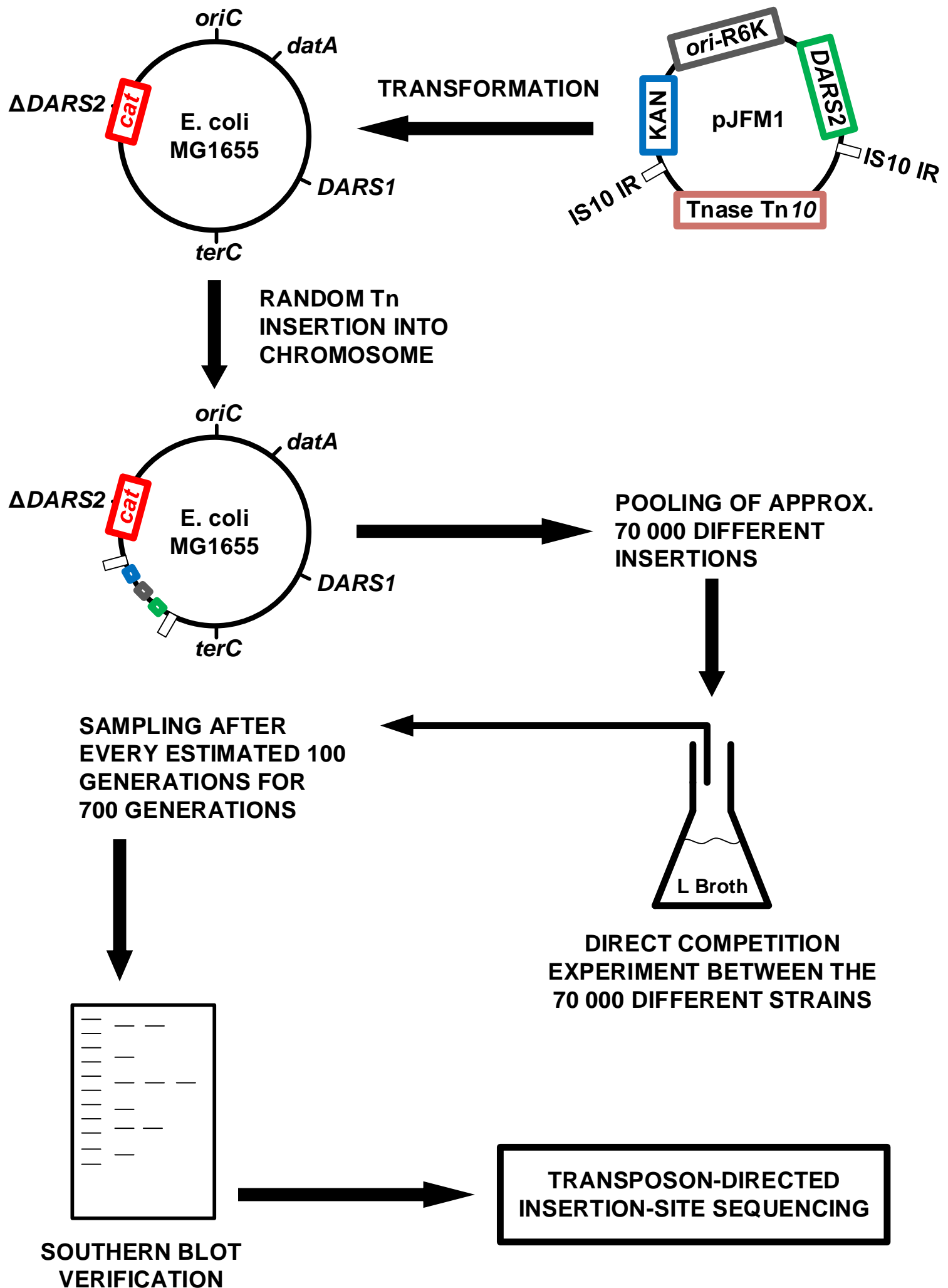
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Figure 1



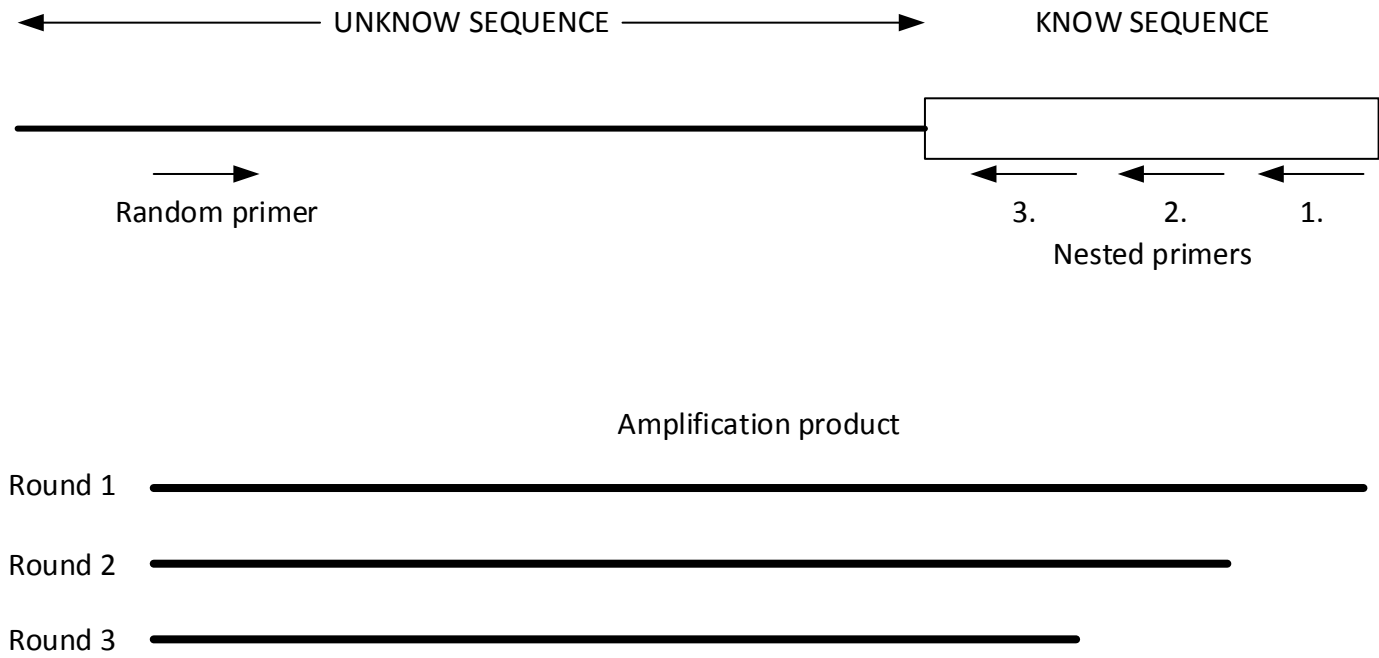
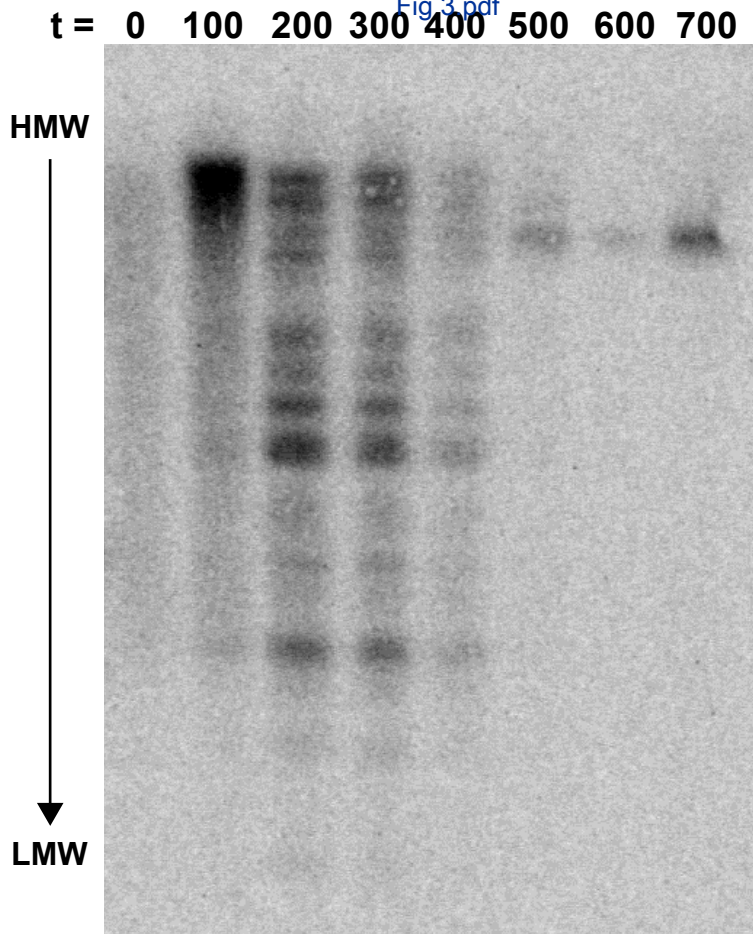
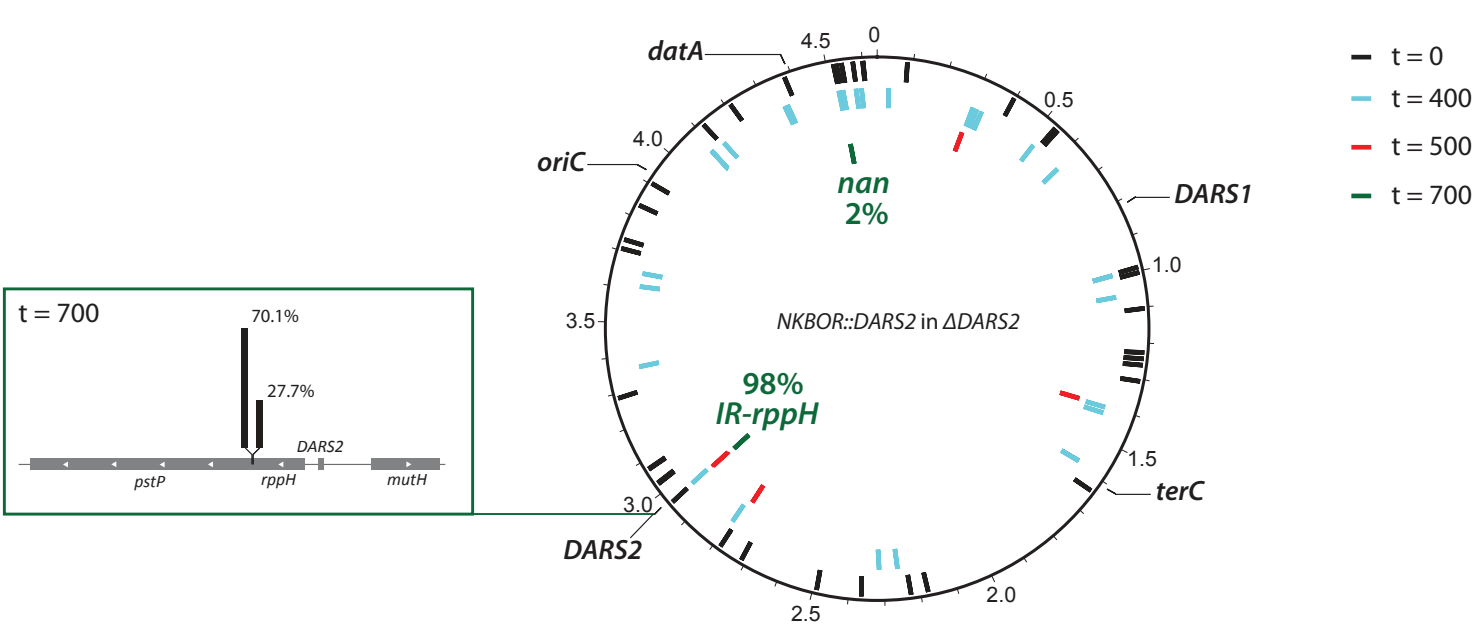
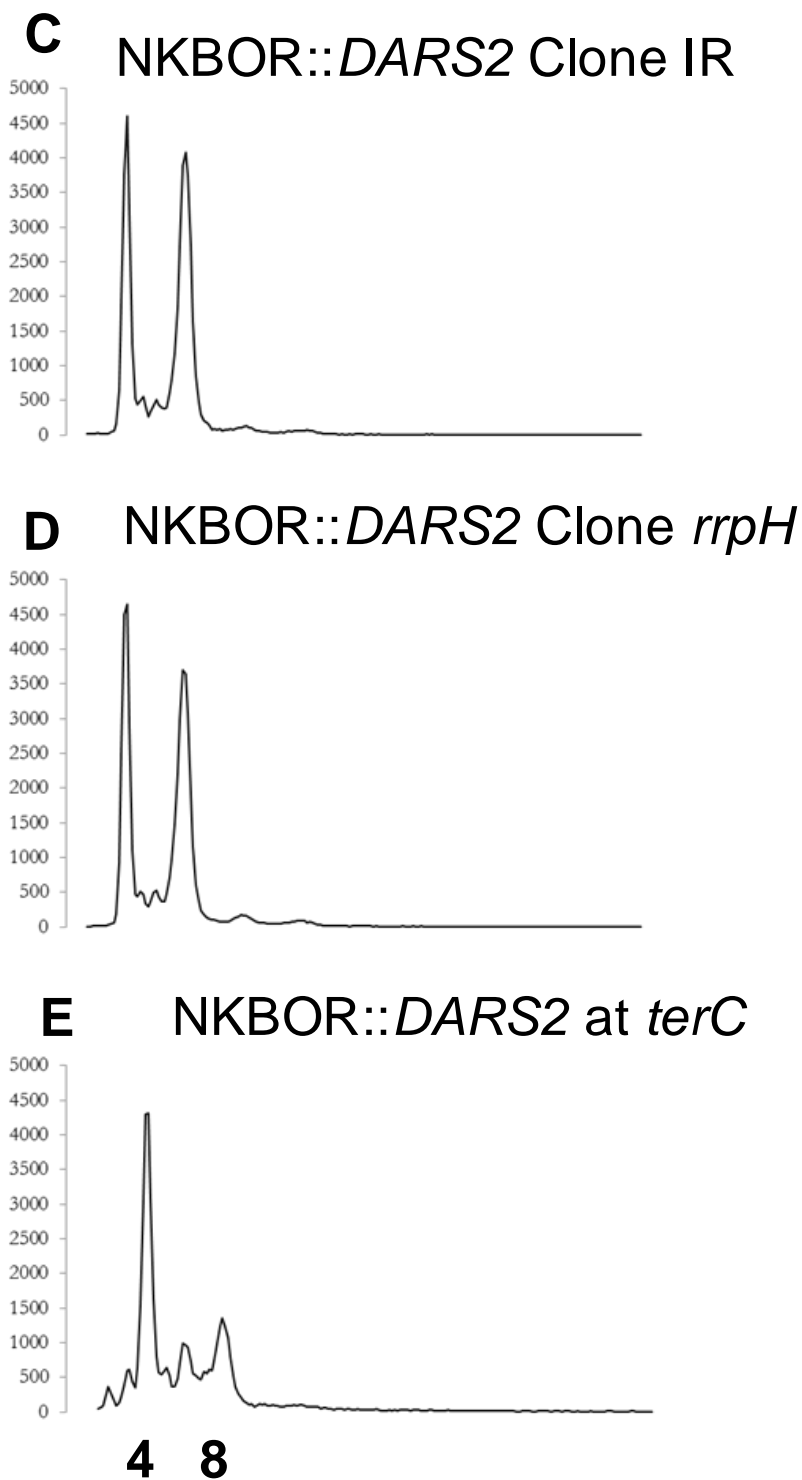
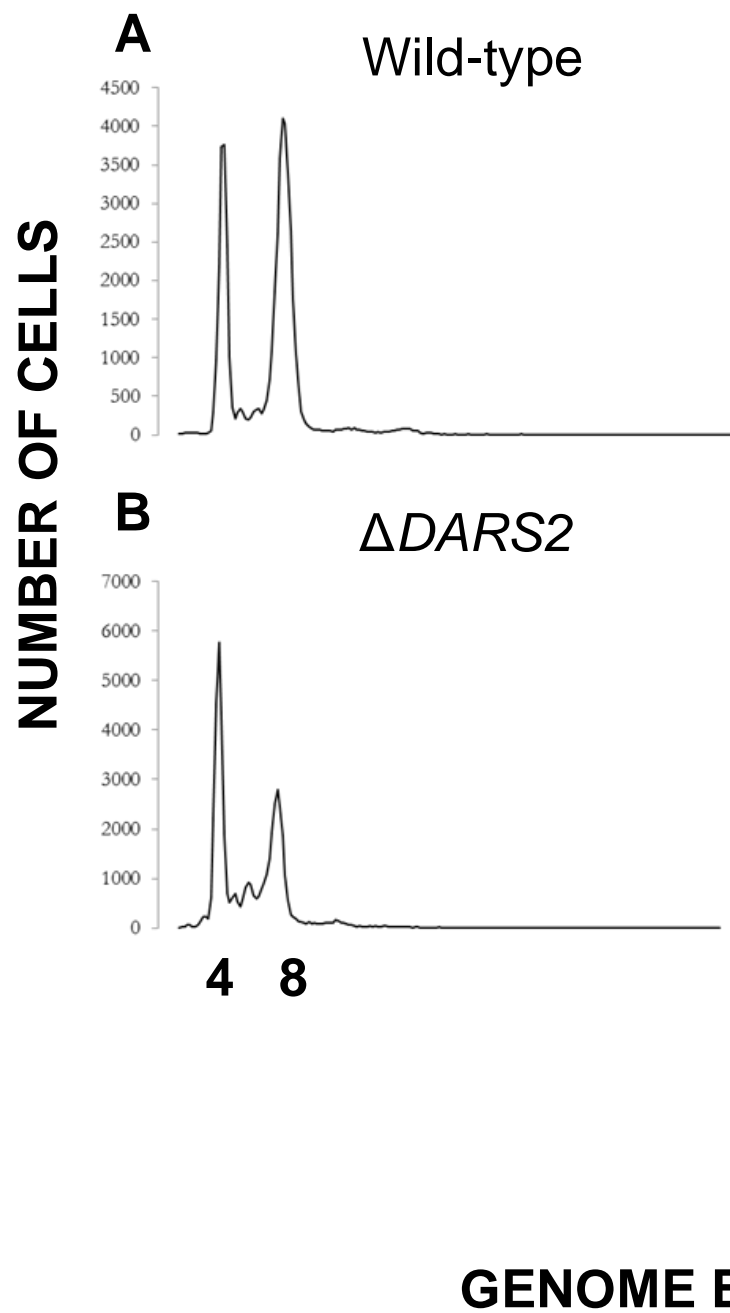


Figure 3

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Autoclaved Mili-Q water		None	
Electroporation Cuvettes, 0.1 cm	Thermo Fisher Scientific	P41050	
Bio-Rad MicroPulser Electroporation System	Bio-Rad	165-2100	
LB Broth	Thermo Fisher Scientific	12780029	
LB Agar, powder (Lennox L agar)	Thermo Fisher Scientific	22700025	
Glycerol	Thermo Fisher Scientific	17904	
Fisherbrand Plastic Petri Dishes	Fisher Scientific	S33580A	
Falcon 50mL Conical Centrifuge Tubes	Fisher Scientific	14-432-22	
Falcon 15mL Conical Centrifuge Tubes	Fisher Scientific	14-959-53A	
Nunc CryoTubes	Sigma-Aldrich	V7634	
Phusion High-Fidelity DNA Polymerase (2 U/ μ L)	Thermo Fisher Scientific	F530S	
dATP, [α -32P]- 3000Ci/mmol 10mCi/ml, 250 μ Ci	PerkinElmer	BLU012H250UC	
DECAprime II DNA Labeling Kit	Thermo Fisher Scientific	AM1455	
Spectrophotometer SF/MBV/03.32	Pharmacia		
Hermle Centrifuge SF/MBV/03.46	Hermle		
Ole Dich Centrifuge SF/MBV/03.29	Ole Dich		
Eppendorftubes 1.5 mL	Sigma-Aldrich	T9661	
Eppendorftubes 2.0 mL	Sigma-Aldrich	T2795	
Sodium Chloride	Merck	6404	
96% Ethanol	Sigma-Aldrich	16368	
Trizma HCl	Sigma-Aldrich	T-3253	
Phenol Ultra Pure	BRL	5509UA	
Chloroform	Merck	2445	
Ribonuclease A type II A	Sigma-Aldrich	R5000	
Sodiumdodecylsulphate (SDS)	Merck	13760	

Lysozyme	Sigma-Aldrich	L 6876
Isopropanol	Sigma-Aldrich	405-7
0.5M Na-EDTA pH 8.0	BRL	5575 UA
Kanamycin sulfate	Sigma-Aldrich	10106801001
PvuI (10 U/μL)	Thermo Fisher Scientific	ER0621
UltraPure Agarose	Thermo Fisher Scientific	16500500
DNA Gel Loading Dye (6X)	Thermo Fisher Scientific	R0611
Tris-Borate-EDTA buffer	Sigma-Aldrich	T4415
Ethidium bromide	Sigma-Aldrich	E7637
Hydrochloric acid	Sigma-Aldrich	433160
Sodium Hydroxide	Sigma-Aldrich	71687
Whatman 3MM papers	Sigma-Aldrich	WHA3030931
SSC Buffer 20× Concentrate	Sigma-Aldrich	S6639
Amersham Hybond-N+	GE Healthcare	RPN119B
Ficoll 400	Sigma-Aldrich	F8016
Polyvinylpyrrolidone	Sigma-Aldrich	PVP40
Bovine Serum Albumin - Fraction V	Sigma-Aldrich	85040C
Deoxyribonucleic acid sodium salt from salmon testes	Sigma-Aldrich	D1626
Carestream Kodak BioMax light film	Sigma-Aldrich	Z373494
GenElute Gel Extraction Kit	Sigma-Aldrich	NA1111
GenElute PCR Clean-Up Kit	Sigma-Aldrich	NA1020
T100 Thermal Cycler	Bio-Rad	
SmartSpec Plus Spectrophotometer	Bio-Rad	
Rifampicin	Serva	34514.01
Cephalexin	Sigma-Aldrich	C4895
Mithramycin	Serva	29803.02
Magnesium chloride hexahydrate	Sigma-Aldrich	246964

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- **Protocol Language:**

Q1) Editor re-wrote some of your protocol steps in the imperative tense. Some non-action items were made into notes.

A1) Ok

Q2) Editor split some of the longer steps.

A2) Ok

Q3) Lines 231-236: Please rewrite this as a step, use a note if necessary, however a section cannot exist without a step.

A3) This has now been rewritten to include steps.

Q4) Section 5: Descriptive sections of the protocol can be moved to introduction, Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.

A4) The previous descriptive section has now been partly moved to the introduction and reformatted to notes and/or incorporated into the steps in the protocol.

- **Protocol Detail/highlighting:** Please add more details to the following protocol steps. For ALL highlighted step, simply citing a references is not sufficient, and explicit stepwise details are required.

All protocol steps have been addressed except for question 19 and 20.

Q19) 5.4.: Are the cells previously counted? How?

A19) The cells have not previously been counted but The cells have not previously been counted , but the flow cytometer ' counts' them during analysis..

Q20) 5.4.1: Please expand and describe using stepwise details about the flow cytometry process. Please mention what button is clicked on in the software to do this, or which menu items need to be selected.

A20) The section has now been expanded to include more details. However we have not expanded details on the software as each make of Flow cytometer instrument and software is unique. We have therefore kept this part general.

- **Figure/Table Legends::**

Q1) Fig 1: Please expand the legend to be self-sufficient.

A1) This has now been added.

Q2) Fig 3: Can a scale of molecular weights be provided?

A2) We do not have a MW scale for this precise experiment. The molecular weights are in this case not important because the southern blot is used to track if the distribution of bands becomes more and more clonal over time. In addition, the single junctions are later sequenced for higher resolution.

- References:

Q1) Please use superscript citation format to move the http link to the reference list (Line 103)

A1) Done

- Please define all abbreviations at first use (LB, NKBOR, PCR, etc)

Done. However, NKBOR is not an abbreviation but the real name of the transposon.

- After the Discussion, please add an “Acknowledgments” section. Please list acknowledgments and all funding sources for your work in this section.

Done

- Please add a “Disclosures” section to your manuscript below the “Acknowledgments” section. In this section, please provide information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included. Please see JoVE’s instructions for authors for more information.

Done