Proof of concept continuous event logging in living cells

Andrey Shur, Richard M. Murray

Abstract

Biological records are omnipresent in paleontology, history, and climate science. Tree rings and ice cores provide evidence of environmental conditions that have been recorded in the composition of materials that are deposited over time, carrying with them a record of events that have influenced their existence before being buried underneath ice or inside the trunk of a tree. We constructed a proof of concept synthetic circuit that can be used to create a similar chronological record of events in the DNA of a living *E. coli*. In our system, phage-based serine integrases are employed to sequentially integrate pieces of DNA corresponding to which stimulus is being detected. We show that placing attB and attP sites close together on a piece of DNA prevents intramolecular reactions, and enables repeated integration events to expand a genetic locus proportionally to integrase induction and abundance of plasmid DNA. We also show that dCas9 binding can prevent integrase from reacting with an attachment site, and in so doing we can control which piece of DNA is integrated by the induction of different guide RNAs. These results represent significant steps towards an event logger that is capable of recording the ordering and magnitude of any number of molecular events. Such a system may be useful in studying complex biological phenomena such as biofilm formation, quorum sensing, or signaling in the gut.

Introduction

Living cells are capable of detecting and responding to sophisticated stimuli present in their environment. Light [1], heat [2], chemicals, and proteins [3] represent a few of the types of stimuli that cells can distinguish. The ability to detect these stimuli is useful to the cell's survival if the cell can appropriately respond to the stimulus; producing a heat-shock protein in response to heat, for example. A biologist, however, must build sophisticated instruments to measure the same stimuli in order to know the magnitude and chronological order of the events that a cell has lived through. In this work, we describe a system that can create a record of chemical stimuli a cell has seen within that cell's DNA.

Previous work on DNA-based event detectors has focused on using phage integrases to irreversibly "flip" pieces of DNA in response to stimuli [4]–[6]. Phage integrases are extremely useful proteins to employ in this regard because their action to recombine specific DNA sequences is deterministic, fast [7], and irreversible. However, integrase-based event recorders typically have a limited number of attainable DNA states, meaning that only a few events can be recorded before the memory capacity is 'used up'. Previous work using cas9 to stochastically excise memory units (conceptually similar to flipping memory units with integrases) in mouse stem cells [8] has shown that a limited number of memory units can be used to record the order and identity of cellular

events over many generations. However, lineage information or cellular events can only be recorded until all memory units have been excised or flipped, which places a fundamental limit on such event recording systems.

The CRISPR system represents a natural chronological record [9] of stimuli where pieces of DNA corresponding to phages are inserted into the genome in the order in which the phages were encountered. Phage genomes are chopped into short oligos, which get inserted into the front of the CRISPR array through the action of the Cas1 and Cas2 proteins. In so doing the CRISPR system keep inserting more phage sequences and continue extending the CRISPR array indefinitely, in contrast with more limited integrase-based memory. More recently encountered phages appear closer to the promoter at the front of the CRISPR array, thus those guides are produced in greater abundance than older guides that reside farther down the array. This allows the cell to focus its immune defenses against more pressing threats, while eventually forgetting the faces of long-vanquished foes.

Several groups have endeavored to harness this recording system to encode the presence of electroporated oligos [10] and chemical stimuli [11] in a “DNA tape recorder” type of circuit that takes advantage of random spacer acquisition during overexpression of the cas1-2 proteins. In particular, Sheth et al [11] have developed a very interesting circuit that utilizes an inducible copy number plasmid to convert a chemical stimulus into DNA abundance, which is in turn reflected in the identity of “random” DNA spacers acquired in the CRISPR array. Using this system, Sheth et al can identify the presence/absence and chronological order of three different chemicals over a period of four days.

We sought to design a DNA tape recorder system that was not dependent on the slow and random activity of cas1-2 proteins to accomplish a similar goal of constructing a DNA array containing a chronological record of events. Our event logger consists of three components (Fig. 1 A): a set of “data plasmids” serves as a source of DNA for integration, taking advantage of plasmid replication to maintain a pool of un-integrated DNA, a synthetic genetic network utilizing phage-based serine integrases serves as the control system which converts stimulus detection into plasmid integration, and an engineered genomic integration site allows for simplified extraction and purification of the integrated fragments for sequencing and read-out.

We believe that our system offers several advantages over that developed by Sheth et al. First, phage integrases are much more efficient and their recognition site is more well-defined than that of cas1-2, which allows our system to react faster than cas1-2 while being less toxic, since phage integrases will not interact with the *E. coli* genome if their cognate attachment site is not present. Second, our system can allow integration of any size of DNA fragment, which can lead to wider applications such as stimulus-directed pathway assembly or programmed integration of promoters and other active genetic elements. We envision these systems being used to produce “molecular sentinels”—bacteria that can be seeded in a river or a waste treatment plant or a gut

microbiome to record chemicals or hormones present over time in a much less obtrusive way than using conventional means.

Results

Serine integrases will catalyze a recombination reaction between attP and attB sites, converting these into unreactive attL and attR sites [12]. To allow repeated integration into the same site, a data plasmid must contain both attP and attB sites, to replace the attB site which is destroyed by the recombination. This presents a challenge because intramolecular attachment sites may be recombined with much higher efficiency than intermolecular sites, meaning that data plasmids will be consumed in non-constructive reactions faster than they can be integrated into the genome. We determined that placing parallel attachment sites closer than 100 bp, as measured from the edge of the attB and attP sequences, decreases the rate at which intramolecular recombination occurs at a rate inversely proportional to the distance between the sites (Figure 1). The minimum intramolecular integration rate occurs at 0 bp spacing, resulting in less than 5% of the intramolecular integration activity seen with 100bp spacing.

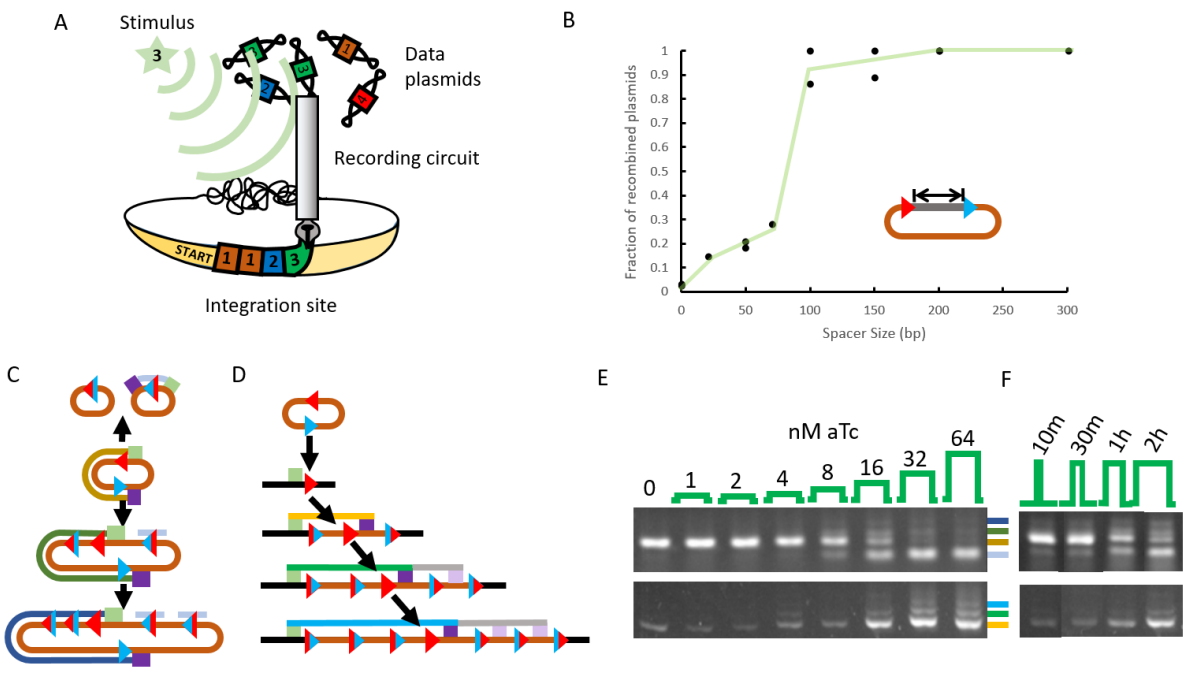


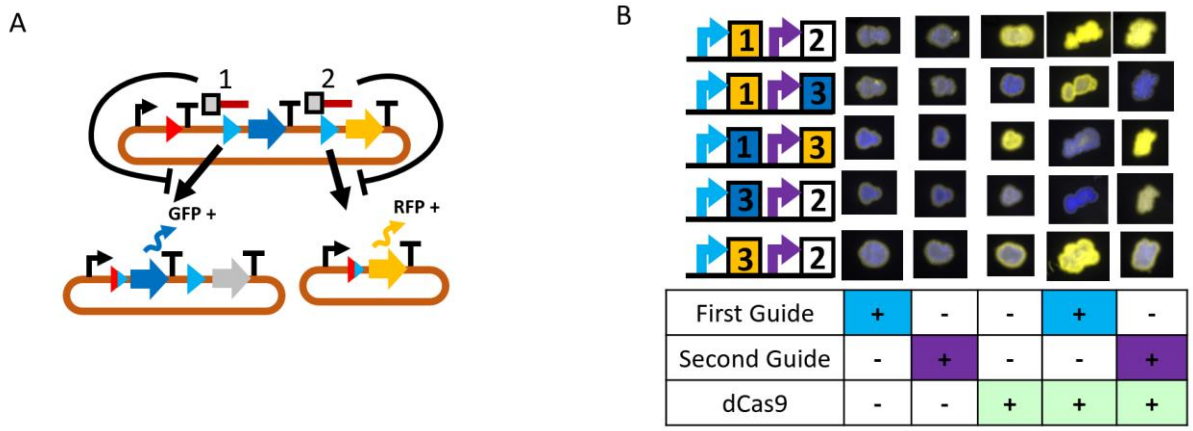
Figure 1: **A**) conceptual representation of the genome event logging circuit. Data plasmids are selected by the recording circuit to be inserted into the integration site, directed by an external stimulus. **B**) Minimal site spacing required for intramolecular integration. Plasmids containing a variable length spacer are incubated with integrase-expressing plasmid in cell-free extract. After incubation for 20 hours, plasmids are purified and transformed into competent cells such that each cell gets one or zero plasmids. Recombined plasmids yield green colonies, while un-recombined plasmids do not express GFP. Colonies were counted

and the results are plotted. Green line is purely for visualization. **C**) Possible integrase reactions that affect the data plasmid. Data plasmids can undergo an intramolecular reaction (up arrow) aka deletion, or intermolecular reaction with other data plasmids, aka multimerization. Green and purple squares represent PCR primers used to detect the presence of deleted or multimerized plasmids. Colored lines represent the PCR product that is obtained, with the length increasing from yellow to green to blue. **D**) Data plasmid can integrate into the genome, yielding a repetitive DNA region. Again, PCR primers are designed such that they produce a longer product the more copies of the data plasmid are inserted. Faint violet and grey lines represent copies of the primer sites located further downstream in the genome site. **E**) PCR performed on liquid cultures of bacteria expressing integrase under an inducible promoter. Upon addition of different concentrations of aTc for one hour, data plasmids are recombined to form multimers (top) and the genome site is extended (bottom). Colored lines at the side of the plot indicate which PCR product (depicted in C and D) is seen. **F**) Same as E, but 16 nM of aTc were added for different amounts of time, after which the cells were spun down and resuspended in fresh media without inducer. A similar trend is observed, with more total amount of inducer resulting in more genome integration and more data plasmid multimerization.

Next, we constructed a proof of concept event logger system consisting of a single data plasmid and tetracycline inducible Bxb1 integrase. Upon induction, integrase was able to catalyze genome integration and data plasmid multimerization in vivo, proportional to the strength of the inducer pulse seen by the cells (Figure 1). Only one hour of induction with aTc concentations ranging from 1-64 nM generated different amounts of integration, with 32 and 64 nM resulting in nearly complete integration (intermolecular or genomic) of all data plasmids. We sought to control the population of data plasmids by arranging the integrase attachment sites in a translational fusion with chloramphenicol resistance. Thus, data plasmids inserted into the genome or multimerized would not be producing a functional antibiotic resistance gene, and the cell would be forced to maintain a population of un-recombined data plasmids to allow for recording future stimuli. However, multimerized data plasmids were not seen to decrease in number even after additional culturing of the cells for 12 hours following the application of the inducer pulse (data not shown). In addition, entire plasmids were integrated into the genome with this system, resulting in multiple functional Cole1 replication origins being present in the genome following integrase induction. We were unable to isolate cells containing different numbers of genome integrated plasmids, possibly because these high copy origins were resulting in polyploidy.

We also wanted to allow recording of the chronological order and duration of multiple events. To this end, the integrase must select between identical attachment sites to integrate different data plasmids into the genome. We have previously shown that catalytically inactive CRISPR-Cas9 could be used to bind and prevent Bxb1 integrase from binding to specific attachment sites in cell-free extract [13]. Now we have shown that this system works in live *E. coli* (Figure 2). A plasmid containing two integrase attachment sites can be made to preferentially integrate one or the other, by co-expression of dCas9 and the appropriate guide RNA. This behavior is dependent on dCas9 expression, but a slight leak of pLac-driven guide RNAs results in colonies

nominally expressing only dCas9 to appear similar to dCas9 and second (pLac) guide RNAs in this experiment. As a proof of principle that this system is sensitive to induction magnitude, we also tried activating guide RNA and dCas9 production in advance of integrase production, to see if pre-production of guide RNA and dCas9 complexes can affect the magnitude of the attachment site repression effect (Figure 2 C). We found that pre-incubation with inducers can increase the magnitude of site selection by about two fold by pre-incubation for 100 min.



C

Expected	0				20				40				60				80				100 min			
0	1	0.5	0.5	69	360	209	167	53	350	299	127	50	363	400	137	42	399	521	150	52	328	460	118	68	346	514	115
1	0	1	0	259	122	332	44	175	124	369	45	139	90	410	46	141	72	433	48	125	63	409	49	150	73	500	28
0.5	0.5	0.5	0.5	120	293	299	103	102	250	326	79	86	187	421	71	78	151	463	72	69	144	410	56	74	122	440	46
0.5	0.5	0.5	0.5	159	153	154	191	152	145	157	151	153	142	178	187	124	140	149	178	116	131	128	166	127	153	136	154

Figure 2: **A**) In vivo integrase attachment site selection reporter construct. Blue triangles are attP sites, the red triangle is an attB site. Bent arrow and T represent promoter and terminator, respectively. In the initial configuration, the promoter is blocked by a terminator from making RNA coding for GFP (blue arrow) or RFP (yellow arrow). Upon integrase-mediated recombination with attB and the first attP, the terminator after the promoter is removed and GFP is expressed. Likewise, if the second attP is chosen, the terminator and GFP sequence are removed, allowing RFP to be produced instead. Guide RNAs, represented by grey boxes with orange tails pointing to the 5' end of the guide RNA (PAM sequence is located approximately where the grey box is), can repress integration at either the first or second site. **B**) Constructs with orthogonal guide RNA sequences under control of inducible promoters were induced in the presence of a reporter construct containing specific guide RNA binding sites. Numbered boxes at the left represent the expected color of the colony if that guide RNA is expressed. For example, the first row has the #1 binding site in front of GFP, such that if guide 1 is expressed, the cells should turn red. Consequently, guide binding site #3 is present in front of RFP in the first row, so when guide #2 is expressed, cells should look the same as when no guide is expressed. **C**) Guide RNA and dCas9 were induced some amount of time before integrase was expressed. Each colored square represents the endpoint fluorescence value of a cell culture containing the test constructs and

dcas9/integrase control system. Vertical columns are different constructs, containing different guide RNA sequences or different guide binding sites in the reporter constructs. Horizontal columns have different inducers added; the top row induces the left guide, the second row induces the right guide, the third row induces dcas9 only, and the bottom row has no inducers added. The expected color of each well is represented with the grid at the left. Raw fluorescence values are plotted in the squares, with the color being determined by whether the number falls in the middle or at a minimal or maximal extreme of RFP expression for all wells tested in the experiment. GFP was not measured in this experiment.

We next sought to investigate the feasibility of sequencing as a readout for the memory array content. Since the array will consist of many repeats of known sequences, long error-prone reads should be more than adequate to deduce the identity and order of memory units in the array. We utilized an Oxford nanopore MinION (Oxford, UK) DNA sequencer to sequence PCR products made from genome sites that had data plasmids integrated. For this experiment we utilized a “proof of concept” event logger as described above. In this system, integrase is induced by addition of aTc, and catalyzes data plasmid integration (Fig. 3). Two different data plasmids were tested, but only a single event—that of aTc being present—was recorded.

The first data plasmid (pF1) tested was designed such that chloramphenicol resistance on the data plasmid is abolished once the plasmid is integrated into the genome. This is designed to encourage persistence of the data plasmid after some integration has already occurred, allowing long duration recordings. The pF1 plasmid also contains a low copy SC101 origin, meaning that less data plasmids are present at any given time. When integrase is induced by 50 nM or 100 nM of aTc, on average 2.07 to 3.25 integrations are observed, respectively (Fig 3 B). After integrating, this plasmid no longer produces functional chlor resistance proteins, but it still has a functional origin which probably results in excessive genome replication.

To alleviate the potential genome instability from multiple repeated replication origins present in the same genetic locus, we constructed a second data plasmid (pF2), which utilizes a promoter-less Cole1 origin. Without the native promoter at the front of the Cole1 origin [14], plasmids cannot replicate. An exogenous promoter is provided upstream of the promoter-less origin, flanked by integrase sites. When pF2 integrates into the genome, the exogenous promoter is separated from the promoter-less origin, producing an array of nonfunctional origins in the genomic recording site. This has the advantage of preventing undesired excessive genome replication, but antibiotic resistance is untouched by integration, and after integration has occurred, cells are no longer required to maintain data plasmids. One consequence of the exogenous promoter driving Cole1 origin replication is a greatly increased plasmid copy number[15], since the exogenous promoter is stronger than the native Cole1 promoter. This may be responsible for the increased average integration count at 50 nM aTc of 3.18 when compared to 3.65 with 100 nM aTc (Fig 3 C).

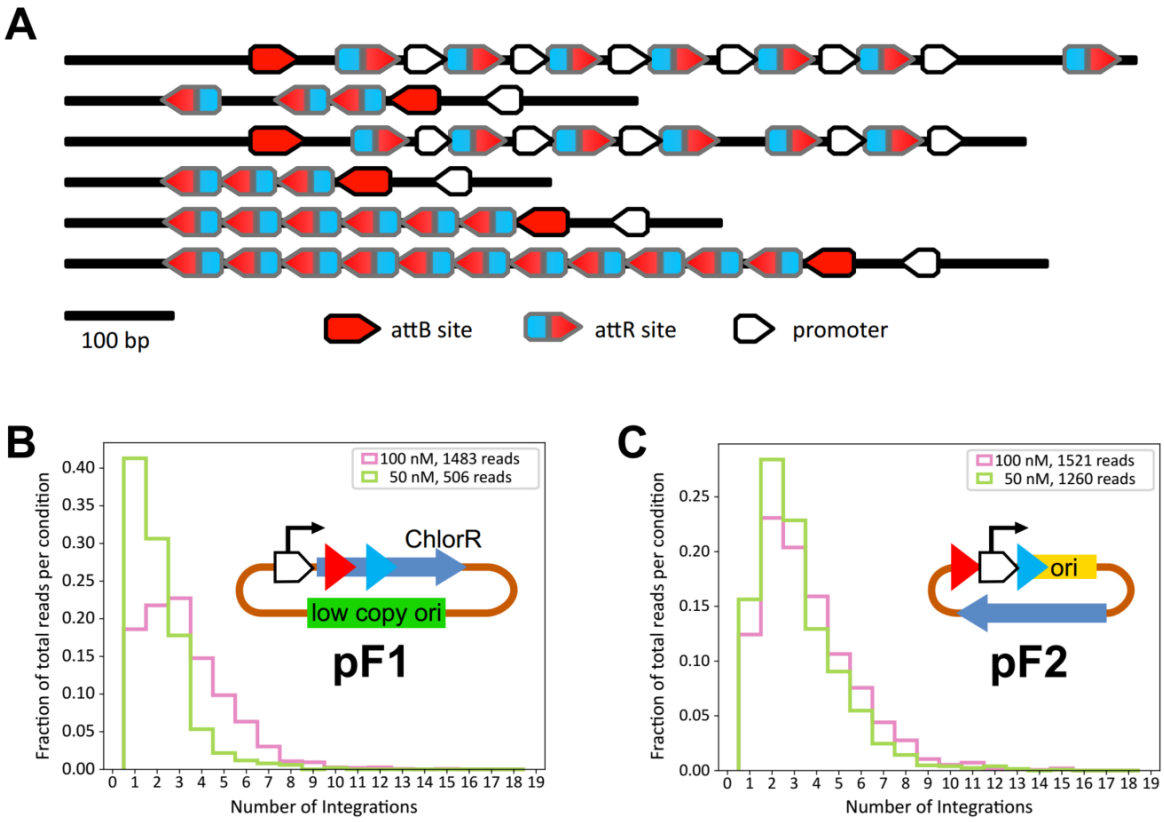


Figure 3. Long read sequencing of multiple integrations. **A)** Representative plot of annotated reads. Each black line represents a single read. Shapes drawn on the line are regions which have > 80% homology to either bxb1 attB, attR, or a constitutive promoter used in the construct. **B)** Cells containing data plasmid pF1 were exposed to 50 or 100 nM aTc, which results in integrase expression from a P_{Tet} promoter in the genome. The pF1 plasmid is maintained at low copy number with an SC101 origin of replication, and has attB and attP sites downstream of a promoter, inside the coding sequence of Chloramphenicol resistance (ChlorR). Average integration site length increased from 2.07 to 3.25 when inducer amount was increased from 50 nM to 100 nM. In this case induction was carried out in minimal M9 media, and inducer concentration was maintained for four hours before cells were lysed and PCRed. **C)** A similar experiment was performed as in B. In this case, pF2 was constructed such that a promoterless Cole1 origin was only capable of plasmid replication before it becomes integrated into the genome. With this plasmid, the distance between attachment sites is greater than in B, yet the average number of integrations observed is approximately similar (3.65 and 3.18 in 100 and 50 nM inducer, respectively).

Discussion

We have developed a proof of concept system that allows tape recorder-like sequential recording of stimuli in a bacterium's DNA. The basic idea is to allow a bacterium to choose between a set of data plasmids to integrate, depending on the

stimuli that are perceived. Integrase attachment sites B and P present on the data plasmids allow continued integration of these plasmids into a single B site in the genome, and selective expression of guide RNAs from chemical sensitive promoters will result in binding and “repression” of attachment site activity, allowing the system to “choose” a data plasmid to insert from a set of possible varieties. In this report we have described a series of steps approaching a complete system capable of genetic recordings, but we are still working to obtain sequences of these repeatedly integrated genome sites. Our goal, of course, is to be able to start with a genome site sequence, and then determine what the sequence of stimuli had to be to obtain such a sequence.

Sheth et al [11] have developed a similar system, taking advantage of the random spacer integration afforded by cas1-2 protein overexpression to integrate portions of a “trigger plasmid”, whose copy number is varied by selective induction of Rep protein expression by chemical sensitive stimulus. The Sheth et al method shares many similarities to the method proposed here, but we believe that our method is more inherently flexible. Since we have decided to utilize serine integrases for our event logger, we can take advantage of the fact that they are capable of integrating large pieces of DNA. There are also many different integrases that have been characterized, with different attachment site specificities to choose from [17]. Thus, our system has greater flexibility in terms of what the final genome array sequence will be. One can imagine a system where entire genes are integrated sequentially, producing a complex operon that is defined by the order of stimuli that a cell has seen. Integrases are known to be quite fast and efficient at recombining DNA, which means we could get away with very low integrase expression, while cas1-2 may have to be driven at expression levels that would stress the cell.

Materials and Methods

Cell strains

Cells used were DH5alpha Z1 from Lutz et al [18]. Genome site constructs were made by Gibson assembly into SpeI-KpnI digested pOSIP KH or pOSIP KO from Pierre et al [19], followed by genome integration and pE-FLP excision protocol as described.

Constructs

Bxb1 integrase sequence was amplified from the Dual-recombinase-controller vector, which was a gift from Drew Endy (Addgene plasmid # 44456) [6]. dCas9 was amplified from pAN-PTet-dCas9, which was a gift from Christopher Voigt (Addgene plasmid # 62244) [20]. Guide RNA sequences were G1: GTTGACcagacaaacccatt, G2: GTTGACcagacaaacctagt, G3: GTTGACcagacaaaccaatg, sgRNA scaffold sequence: GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC. Bxb1 attB: GGCTTGTCGACGACGGCGTGCTCCGTCGTCAGGATCAT, Bxb1 attP: GGTTTGTCTGGTCAACCACCGCGTGCTCAGTGGTGTACGGTACAAACC. Cole1 origin with no promoter was made by PCR with PCOLE1NOP001_F: AAAGGTCTCAGCTTGCAAACAAAAAAACCACCGCTACC, and PCOLE1NOP002_R: AAAGGTCTCACTCCGCCAGGAACCGTAAAAAGGCC,

Experiments

Cells were grown to 0.2 OD in Luria Broth or M9CA minimal media (Teknova) then inducers were added. 1-100 nM Anhydrotetracycline (Sigma), 0.2 uM Sodium Salicylate (Sigma), 0.2% Arabinose (Teknova), 1 mM Isopropyl-beta-D-thiogalactoside (Sigma). Cells were subsequently grown for varying amounts of time. Then, 10 uL of cells were transferred into another 1 mL of culture and cells were grown for 12 hours again.

Sequencing

PCR products for pF1 constructs were obtained using pASS_pF1F: aaaCACCTGCaaaaTTACGGCGTATCACGAGGCAGAAT and pASS_genR: aaaCACCTGCaaaaCCTGGTACAGACAGGAGCTGCGTT. PCR products for pF2 constructs were obtained with pASS_pF2F: aaaCACCTGCaaaaTTACCGGTATCAACAGGGACACC and pASS_genR as above. PCR products were subsequently digested with AarI (NEB), and ligated to barcodes consisting of phosphorylated, dA tailed, annealed oligos having the following sequences:

name	sequence
ASSBc10	GAGTCTTGTGTCAAATTGTACGCCAGTTACCAGG*
ASSBc56	GAGTCTTGTGTCCATCACCTTTCCAGTTACCAGG*
ASSBc126	GAGTCTTGTGTCTGTTTACCGACCAGTTACCAGG*
ASSBc49	GAGTCTTGTGTCATTAGCTGGGCCAGTTACCAGG*

* these barcodes can have a TTAC or CCTG 5' overhang on the right side, and they have a 3'A overhang on the left side so they can be ligated to the nanopore sequencing adapters provided in the MinION ligation sequencing kit.

Following barcode ligation, sequencing prep proceeded as described (1D amplicon by ligation (SQK-LSK108) protocol). Loading on MinION R9.4 flow cell proceeded as per manufacturer recommendations.

Alignment

Sequences were matched to known features using Geneious sequencing analysis software. Known feature sequences were as follows:

name	sequence
AttB	TCGGCCGGCTTGTCGACGACGGCGGTCTCCGTCGTCAGGATCATCCGGGC
AttR	TCGTGGTTTGTCTGGTCAACCACCGCGGTCTCCGTCGTCAGGATCATCCGGGC
Promoter	TTGACAGCTAGCTCAGTCCTAGGTATAATACTAGT

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