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Shuffling of DNA cassettes in a synthetic integron

Summary

The complexity of even small gene networks makes them difficultly amenable to rational design. Testing random combinations of genetic elements in a directed evolution procedure is thus of interest for many applications including metabolic engineering. Here we describe how the recombination machinery of class 1 integrons can be used to deliver and shuffle genetic elements at a chromosomal locus in *E. coli*.

Key words:

Synthetic Biology, Directed Evolution, Recombination, DNA shuffling, Integron, Metabolic Engineering.

1. Introduction

Over the past 20 years, efficient mutagenesis techniques have been developed allowing the introduction of random point mutations, as well as sequence shuffling through homologous recombination. However the efficient random recombination of heterologous sequences largely remains a challenge. It has been achieved *in vitro* through various techniques of randomized assembly ligation and overlap extension PCR (1-5). However these techniques are limited in the size and number of elements they can combine.

Integrons are natural recombination platforms involved in the rapid adaptation of bacteria to changing environment. They are present in the genome of roughly 10% of sequenced bacteria, and are largely responsible for the massive spread of multiple resistances to antibiotics. Integrons consist in an integrase gene (*intI*) able to capture, stockpile and shuffle gene cassettes. Natural cassettes generally consist in a single promoterless ORF associated to an *attC* recombination site. Cassettes can be excised through *attC* x *attC* recombination into a circular intermediate and preferentially integrated at a primary recombination site downstream of the integrase gene called *attI*. Different classes of integrons have been identified on the base of the integrase sequence identity (for a comprehensive review see (6)).

We use here the class 1 integrase isolated from R388 multiresistant integron, the associated *attI1* site, and the *attC_{aadA7}* site isolated from an *aadA7* spectinomycin resistance cassette. These elements have been extensively described and shown to efficiently recombine. We have already shown how this machinery can be used to deliver gene cassettes of the tryptophan operon and shuffle them to generate a wide variety of combination yielding improved phenotypes (7). Here is a detailed procedure of the steps required to construct a strain with a synthetic integron integrated in the chromosome, to deliver new genetic elements in the array and to shuffle them.

2. Materials

The minimal platform of an integron consists in the integrase gene and the *attI* recombination site. In all experiments, the *IntI1* integrase is expressed in *trans* from the pBAD::intI1 plasmid. The *attI1* site is integrated into the chromosome at the *attB* site of bacteriophage lambda using a suicide integration vector (procedure described below). Depending on your application you may want to directly clone cassettes in the integron array that you will integrate into the chromosome.

2.1 Biological material: DNA

1. Plasmid pSWK carrying an *attI* site. The pSWK plasmid carries an R6K origin of replication which depends on the Π protein expressed from the *pir* gene. It also carries the *attP* site from bacteriophage lambda, allowing its integration at the lambda *attB* site in the *E. coli* chromosome. This plasmid complies with biobrick standard RFC10, and can be found on the registry of standard biological parts as BBa_J99000 (7).
2. The integron *attC_{aadA7}* site: BBa_J99001 (7).
3. The integron *attI1* site: BBa_J99002 (7).
4. Plasmid pBAD::IntI1 (8).
5. Plasmid pTSA29-CXI-AK (9). A thermo-sensitive pSC101 vector carrying the lambda integrase under the control of the thermo-inducible CI857/pR promoter.

2.2 Biological material: strains

1. π 1 *E. coli* cells (*DH5 α* *ΔthyA::(erm-pir116)* [EmR]), for the replication of the pSWK plasmid (10)
2. MG1655 *E. coli* cells (or any *E. coli* strain where you want to integrate the synthetic integron)
3. ω 7249 *E. coli* cells (*(F-)* *RP4-2-Tc::MuAnic35 ΔdapA::(erm-pir)* [KmR ErmR]). ω 7249 cells express the *pir* gene allowing the replication of vectors carrying the R6K replication origin. It also expresses the RP4 conjugation machinery allowing the transfer of plasmids carrying the RP4 origin of transfer (11).

2.2 Culture media and reagents

1. Luria Bertani (LB) culture medium
2. LB agar plates supplied with suitable selection agent
3. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μ g/ml, chloramphenicol (Cm), 25 μ g/ml.
4. Thymidine (Thy) and diaminopimelic acid (DAP) is supplemented when necessary to a final concentration of 0.3mM.
5. Glucose and L-arabinose are added at respectively 10 and 2mg/ml final concentration for the repression and induction of the pBAD promoter.
6. CaCl₂ solution, 50uM
7. EcoRI, XbaI, SpeI, PstI restriction enzymes. Those are the restriction sites of the RFC10 assembly standard.
8. T4 DNA ligase

3. Methods

The global idea is to first integrate the *attI1* site into the chromosome. This will be the landing platform of any integron cassette of your choice. Cassettes present in the array can be shuffled through the expression *in trans* of the IntI1 integrase from the pBAD::intI1. Finally, new cassettes can be delivered to the array through a suicide conjugation assay.

Depending on your application an initial cassette array can directly be integrated together with the *attI* site. Cassettes are simply created through the association of any DNA element to an *attC_{aadA7}* recombination site and cloned downstream of the *attI* site. This can be done using the construction method of your choice. The method we use here is biobrick standard assembly. We constructed vectors carrying *attI* (BBa_J99002) and *attC* sites (BBa_J99001) complying with the RFC10 standard.

Several methods have been described for the efficient integration of DNA in the *E. coli* chromosome (12, 13). The method we chose here is to clone the initial integron cassette array on a vector (pSWK) that can then be integrated at the chromosomal *attB* site of bacteriophage lambda. pSWK carries an *attP* site and can thus be integrated at the *attB* site by the lambda integrase. It has an R6K origin of replication and can only replicate in cells expressing the *pir* gene. This plasmid can thus be integrated into *pir*-cell expressing the lambda integrase *in trans* (from plasmid pTSA29-CXI-AK), and plating on the pSWK resistant marker will select integration events. However, any other integration method and any other neutral chromosomal locus should be suitable.

New cassettes can be delivered to an integron array through a suicide conjugation strategy. The pSWK vector can be used to this end too. pSWK carries an RP4 origin of transfer, and can be delivered to recipient cells by ω 7249 cells expressing the RP4 conjugation machinery. This donor strain can be used in a conjugation assay to deliver cassettes in a recipient strain carrying an integron array and expressing the IntI1 integrase *in trans* from the pBAD::IntI1.

3.1 Construction of the initial cassette array

1. The initial integron array can be constructed with the method of your choice. Here, we use a classic digestion / ligation / transformation protocol. The use of the RFC10 idempotent assembly standard allows the easy association of any biobrick to an *attC_{aadA7}* site and its cloning into the integron array on the pSWK vector. For more details on biobrick assembly see partsregistry.org. The pSWK vector can only replicate in cells expressing the *pir* gene. Transformation is thus carried out into pi1 cells, and selection on LB agar + Chloramphenicol (25 microg/ml) + Thymidine (70 microg/ml) plates.

3.2 Integration of the initial cassette array into the chromosome

1. You first need to transform the pTSA29-CXI-AK plasmid carrying the lambda integrase into your strain. The method of your choice can be used to prepare competent cells. We use here a classical CaCl₂ chemo-competent protocol. After heat-shock, cells need to be incubated at 30°C for the expression period

and plated on LB agar + Ap at 30°C in order for the plasmid to stably replicate.

2. In order to integrate your pSWK into these cells, you now need to prepare competent cells expressing the lambda integrase. Launch an overnight culture at 30°C in LB + Ap from a single colony
3. Dilute the overnight culture to the 1/100th in LB and grow at 30°C until OD₆₀₀=0.2
4. Switch the incubation temperature to 42°C until 0.6 < OD₆₀₀ < 0.8 . During this period, the lambda integrase will be expressed from the CI857-PR promoter.
5. Prepare CaCl₂ competent cells (or any other type of competent cells. The integration event is very efficient, so you don't need to prepare ultra-competent cells for this to work). (see note 1)
6. Transform with at least 20 ng of pSWK DNA, and incubate for the expression time for 1H at 42°C.
7. Plate on LB agar + Cm and grow overnight at 42°C. Doing all this steps at 42°C ensures that the lambda integrase is expressed and that at the same time the pTSA29 is lost.
8. The following day you should have colonies with the pSWK integrated at the *attB* site of your strain.
9. Check for proper integration by doing a colony PCR using a primer binding on the pSWK and one binding on the chromosome.

3.2 Cassette shuffling through the IntI1 integrase expression

1. Transform the pBAD::IntI1 in your cells using the method of your choice.
2. Induce the integrase by diluting an overnight culture to the 1/100th in LB + Ara. You can prepare a negative control in LB + Glu. Grow overnight (see note 2)
3. Plate cells on the selective media of your choice. Recombinations induced by the integron machinery happen at frequencies below 10⁻². In order to find recombined clones, you need a powerful screening assay. In our initial study where we shuffle the genes of the tryptophan biosynthesis operon, the selection is performed in tryptophan auxotroph strains and recombinants are selected on minimal media (7).

3.3 Delivery of new cassettes through conjugation

1. Transform the pSWK carrying the cassettes of interest into ω 7249 cells. We use CaCl₂ competent cells, but any method is suitable. Remember that ω 7249 cells need DAP to grow. (see note 3)
2. This strain will be your donor strain in the conjugation assay. Depending on your application you may want to use several donor strains to combinatorially deliver cassettes of interest in a single recipient strain. The recipient strain needs to carry the pBAD::intI1 plasmid and an *attI* site in the chromosome. Dilute an overnight culture of the donor strain(s) to the 1/100th in LB + DAP and of the recipient strain in LB+DAP+Ara (see note 4).
3. Grow donor and recipient cells to 0.4 < OD₆₀₀ < 0.6
4. Mix 1ml of donor cells with 4ml of recipient cells and filter onto a 0.22um membrane. (see note 5)

5. Place the filter on a fresh LB+DAP+Ara plate, the side with the cells facing up. Incubate 6H at 37°C.
6. Put the filter in a 50 ml tube with 5 ml LB and vortex to remove the cells from the filter.
7. Plate the cells on the selective media of your choice. (If the pSWK vector you use carries a resistance that is not already present in your recipient strain, you can select for the integration of the whole vector in the integron array. It is then possible to get rid of the vector backbone using a counter selection marker, leaving all or some of the cassettes in the chromosome.)

4. Notes

1. It is important that competent cells are kept on ice throughout the whole process.
 2. The longer the cells are growing with the integrase expressed, the higher the recombination frequency will be. To improve recombination frequency, several passages in LB+Ara can be done. Note however that without selection, cassettes are lost at a frequency similar to the one of reordering events (7).
 3. The use of the DAP auxotroph donor (ω 7249) for the conjugation delivery protocol guaranties that you get rid of the donor by simply selecting on LB + antibiotic marker of the pSWK, as DAP is completely absent in LB.
 4. The recipient strain does not actually need DAP to grow, but adding DAP in the medium allows to later mix the cultures without worrying about killing the donor cells
 5. If you do not have the equipment to do this, you can mix the cells and centrifuge them gently 4min at 2000g, discard the supernatant, resuspend the cells in 100 microl and pipette onto the filter.)
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