Direct Cloning and Heterologous Expression of Biosynthetic gene cluster

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Part 1. Transformation Associated Recombination (TAR) Based Direct Cloning of Biosynthetic Gene Cluster

Materials

Yeast strain

A highly transformable *S. cerevisiae* VL6-48N (**not VL6-48**) strain (MAT α, *his3-D200*, *trp1-Δ1*, *ura3-Δ1*, *lys2*, *ade2-101*, *met14*, *psi+cir^{O}*) can be used as a host for gene cluster direct cloning experiments (a gift from Dr. Vladimir Larionov, National Cancer Institute, National Institutes of Health, Bethesda, USA). The yeast cells were grown in liquid Yeast extract Peptone Dextrose medium (YPD medium: 2% D-glucose, 1% yeast extract, and 2% peptone), supplemented with 100 mg/L adenine (adenine is not necessary for cells growth but is recommended for the direct cloning experiments) and used for spheroplasting.

Grow them on YPD agar plate supplemented with 100mg/l adenine and store the plate in 4℃ until use.

TAR cloning solutions

1. 10x nitrogen bases (100ml)

1.7g of Yeast Nitrogen Base Without Amino Acids and Ammonium Sulfate (Sigma Y1251)

1.9g of Yeast Synthetic Drop-out Medium Supplements without tryptophan (Sigma Y1876)

5g of Ammonium Sulfate

Disolve above components in 100ml warmed up Milli-Q water and filtrate with 0.22um filter to make x10 stock sol (**store at 4 ºC**).

2. 100x adenine (50ml): 0.5g adenine, add 3.7ml of 1M HCl

3. Top selective agar (50ml, for 6 transformations)

Sorbitol	9.1g, 1.1M
Dextrose	1.1g, 2.2% (w/v)
Agar	1.5g

*Before using, microwave it and add 10x N-bases and 100x adenine

4. **Bottom selective agar (500ml)**

Sorbitol	91g, 1.1M
Dextrose	11g, 2.2% (w/v)
Agar	10g

*Before using, microwave it and add 10x N-bases, 100x adenine and **0.0001% 5-fluoroorotic acid (5-FOA, dissolved in DMSO 100mg/ml 100X stock solution)**

5. Liquid YPD medium (Yeast extract peptone dextrose medium, 500ml)

Yeast Extract	5g
Peptone	10g

Autoclavable, then add glucose to 2% (w/v) final

6. YPD agar (500ml)

Yeast Extract	5g
Peptone	10g
Agar	7.5g

Before using, microwave it and add glucose to 2% (w/v) final

7. Glucose, 40% (w/v)

8. 500ml 1M Sorbitol (91g)

9. Milli-Q water, 200 ml

10. HEPES buffer, 1M and pH 7.5

11. EDTA Solution, 0.5M, adjust the pH to 8.0

12. 100mM, $CaCl_2$ (1.1g in 100ml water)

13. SPE (50ml)

HEPES Buffer (11)	500ul
EDTA Solution	1ml
Sorbitol	9.1g
Water	Up to 50ml

Autoclavable and store up to 2 months at room temperature

14. SOS in 50ml

CaCl2 solution (13)	3.25ml
Yeast extract (0.25%)	0.125g
Sorbitol	9.1g
Peptone	0.5g
Water	Up to 50ml

Autoclavable and store up to 2 months at room temperature

15. Tris Buffer, 1M and pH7.5

16. STC (50ml)

Tris buffer, 1M (15)	500ul
$CaCl_2$ solution (13)	5ml
Sorbitol	9.1g
Water	Up to 50ml

Autoclavable and store up to 2 months at room temperature

17. PEG (10ml, pH7.5)

Tris buffer, 1M (15)	100ul
$CaCl_2$ solution (13)	1ml
PEG8000	2.0g
Water	Up to 10ml

Filtrate and store up to 4 weeks at room temperature

18. Zymolyase 20T; 10 mg/ml zymolyase-20T (MP Bio), 25% (w/v) glycerol, 50 mM Tris-HCl (pH 7.5). Dispense into 500-µl aliquots, and store up to 2 years at −20℃.

Capture vector pCAP03-*acc(3)IV*

(A counter-selectable yeast centromeric-*E.coli* shuttle and Actinobacterial chromosome integrative)

This vector is a derivative of pCAP01. *URA3* gene was introduced into pCAP01 under the strong promoter of the *Schizosaccharomyces pombe ADH1* gene (*pADH1*) into the SpeI and KpnI restriction sites of pCAP01, as a counter selectable marker, to produce pCAP03. *pADH1* can tolerate an insertion of up to 130 bp between the TATA box and the transcription initiation site. This method allows the use of shorter capture arms and conveniently selects against non-homologous end joining in the presence of 5-fluoroorotic acid (5-FOA). In order to easy linearize pCAP03, the 1369 bp apramycin resistance cassette (*acc(3)IV*) was amplified from pIJ773 and cloned into the pCAP03 to obtain pCAP03-*acc(3)IV* (Figure 1). Before assembly of our cluster-specific capture vectors, the circular construct pCAP03-*acc(3)IV* needs to be digested with XhoI and NdeI and agarose gel purified, as ready-to-use pCAP03 (RTU-pCAP03) (Figure 1).

The vector also has the same features as pCAP01, including ARSH4/CEN6-TRP1 (yeast elements from pRS314), pUC *ori* from SuperCos1 (for maintenance in *E.coli*), and φC31 *int-attP-oriT-aph* (*Streptomyces* elements from a pSET152 derivative). For yeast, tryptophan deficient media is used for selection of transformants. Both in *E.coli* and Actinobacteria, 50ug/ml Kanamycin (or 30-50 ug/ml Neomycin) is used as a selective agent. If you need to use a expression host that has *aac(3)IV* (apramycin resistance) gene already or a Kanamycin resistant strain, use 30-50 ug/ml Neomycin for the selection.

This vector is maintained at single copy in yeast cells. This is very important feature to avoid unwanted multiple recombination in yeast. So far, it has been confirmed that huge DNA insert up to 75kb could be cloned and carried stably on this vector (both in yeast and *E.coli*).

Procedure

Construction of specific capture vectors

To construct a pathway-specific capture vector, 144 bp dsDNA fragments were designed as shown in Fig. 2 and synthesized by your local DNA synthesis company specific. The 100 ng *ds*DNA fragments (if you don't have enough *ds*DNA, PCR can be used for propagating) were assembled with 50 ng of the RTU-pCAP03 following the instructions for the Gibson Assembly Kit (New England BioLabs Inc) to generate a pathway-specific capture vector. Prior to direct TAR cloning, the capture vectors were digested by PmeI (column purification is recommended to clear the digested vector).

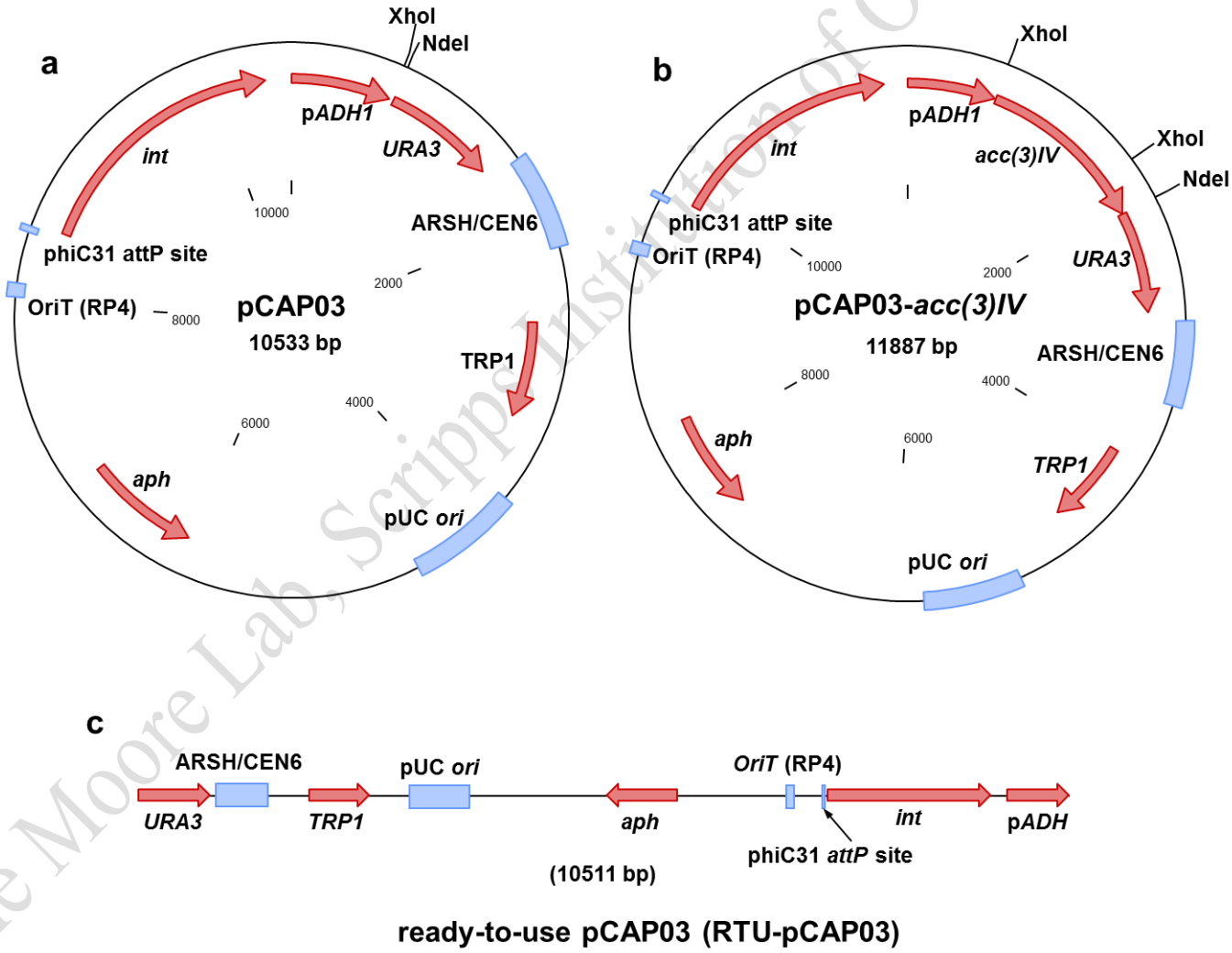

Figure 1. Physical map of the gene cluster capture vector.

(a) pCAP03, (b) pCAP03-*aac(3)IV*, and (c) ready-to-use pCAP03 (RTU-pCAP03)**.**

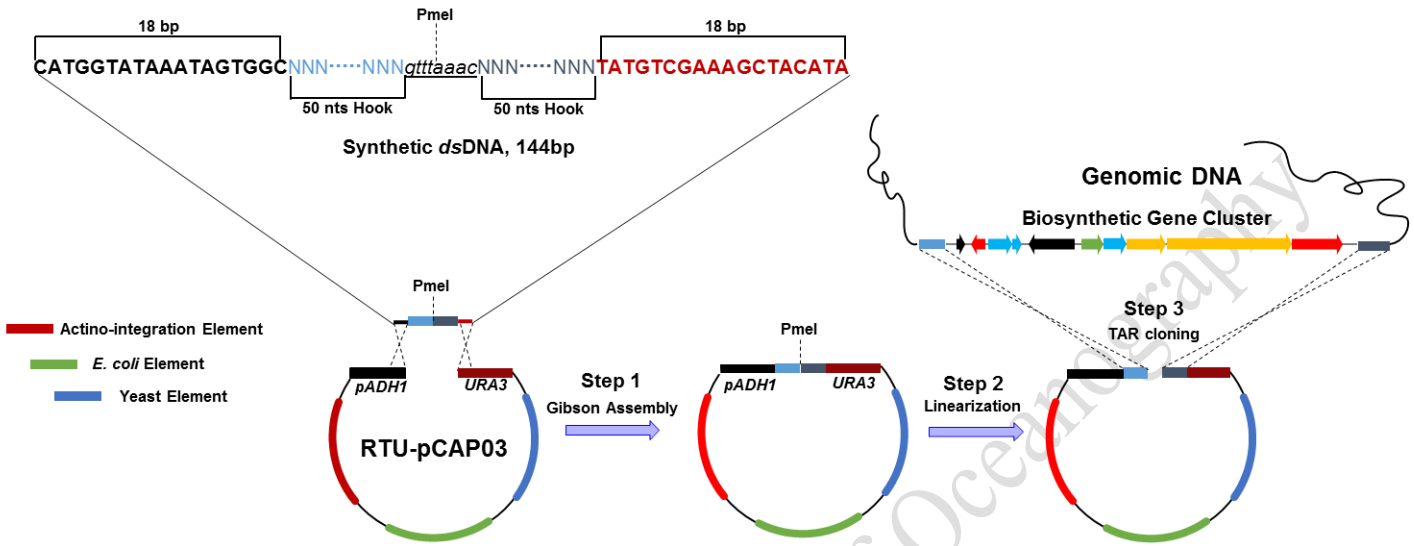

Figure 2. PCR-independent direct cloning strategy for capture of natural product gene clusters. (a) PCR-independent TAR cloning involves three steps. In step 1, the capture vector is constructed in one step by Gibson Assembly using 144 bp synthetic *ds*DNA. In step 2, the capture vector is linearized by the restriction enzyme PmeI. In step 3, recombination in yeast.

Preparation of genomic DNA

In yeast spherolast transformation step, cells are transformed with linearized capture vector and gDNA fragments. gDNA must be fragmented to appropriate size because too large fragment is hardly taken up by the cells. Quality of gDNA fragments (especially in size distribution) also greatly affects recombination efficiency. For isolation of gDNA from antinobacteria, please fellow the protocol from the book “Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. and Hopwood, D.A. (2000) **Practical Streptomyces Genetics**: John Innes Foundation, Norwich Research Park, Colney, Norwich NR4 7UH, UK”

Preparation of yeast spheroplast cells and transformation

Current protocol below has been slightly modified from the original method described in the Nature Protocols paper.

While they prepare yeast spheroplast cells from mid-log phase cells (OD600=3.0-5.0), early-log phase cells (OD600 around 1.0) are used in the modified protocol below since younger cells show higher competency.

To transform yeast cells with huge DNA molecule such as genomic DNA, cell wall must be removed perfectly by a lytic enzyme, Zymolyase, in prior to transformation. However, too much Zymolyase treated cells are hardly regenerated because spheroplast cells are extremely unstable. The original protocol doesn't have osmotic stabilization step before spheroplasting, and thus they could digest cell wall only partially with the enzyme (<80% conversion). By adding stabilization step before spheroplasting, Zymolyase treatment can be more perfectly done (nearly 100% conversion) without cell lysis. By these modifications, now we can transform yeast cells with huge DNA molecules at a high efficiency.

Basic transformation protocol

Day 1

1. Grow a single colony of *S. cerevisiae* VL6-48N strain in YPD liquid medium containing adenine (~10ml) overnight at 30 ℃ with shaking. **Colonies grown on YPD pates can be kept at 4℃ for up to 1 week**.

Day 2

2. Inoculate 0.5-1ml yeast cell from overnight culture in 50ml YPD media contianing adenine (in 250ml-flask). Grow for 5-8 h at 30°C shaking at 220 rpm to an OD600 of ~ of **0.7-1.0**. This will give you enough early-log phase cells for 10 transformations (growth phase of the cell greatly affects transformation efficiency).
3. Put the flask on ice for 10min and then centrifuge the cells in a 50 ml falcon tube for 3 min at 1800×g, 4℃.
4. Decant supernatant and resuspend the pellets in 50 ml ice-cold sterile water by vortexing and then centrifuge for 3 min at 1800×g, 4℃.
5. Decant water and resuspend the cells in 50 ml of 1 M ice-cold sorbitol by vortexing. **Leave the cells on ice (or 4℃) overnight for osmotically**.

Day 3

6. Invert the tube several times and then centrifuge for 3 min at 1800×g, 4℃.
7. Remove the supernatant completely by standing the tube upside down on a paper towel, and resuspend the cells in 20 ml SPE solution (room temperature) by vortexing. Add 40µl of 2-mercaptoethanol (2-ME), and invert to mix. Then add **80µl zymolyase-20Tsolution** and invert to mix.
8. **Incubate 40 min in 30℃ incubator** (invert the tube every 5 min or use shaker with slow speed).
9. Check the level of spherolasting by measuring the optical density (at 600nm) of cell suspension. Dilute zymolyase treated cells 5 times with 1M sorbitol and 2% SDS (in water). Measure optical density at 600nm of both suspensions and compare their ODs. **The spheroplasting are determined to be ready when their difference is five- to twenty-fold (≒80-95% of cells has to be converted to spheroplast).** If the difference is not enough, keep incubating for additional 10min and measure the OD difference again. The level of spheroplasting greatly affects transformation efficiency (insufficiently digested cells do not take up large DNA molecule, but incubation time shouldn't be longer than 50min). You can use a large construct (any pCAP captured gene cluster) as control to qualify the spheroplast.
10. Add 1 M sorbitol (4℃) up to 50 ml and gently invert to mix, and harvest the cells by centrifuging for **10 min at 600×g, 4℃**.
11. Decant the supernatant and stand the tube upside down on a paper towel to remove supernatant completely (cell pellet is not tightly packed! Do not loose cells!).
12. **Gently resuspend the spheroplasts** in 20 ml of 1 M sorbitol (4℃), **by pipetting up and down with a 25-ml pipette**. It takes about 2 min to resuspend the spheroplast cells completely (spheroplast cells are extremely unstable. Do not vortex for resuspending). Add 1 M sorbitol (4℃) to a total volume of 50 ml, and invert to mix.
13. Harvest the cells by centrifuging for **10 min at 600×g, 4℃**. Remove supernatant completely by standing the tube upside down on a paper towel.
14. Resuspend the spheroplasts in 2-3 ml STC solution (room temperature) by pipetting up and down with a 10-ml pipette (it takes about 2 min to resuspend the spheroplast cells completely).
15. Incubate the spheroplasts 10 min at room temperature (22℃).
16. Mix 200 µl spheroplasts with the ≤40 µl transforming DNA solution (0.2-0.5ug linearized vector and 2-3ug gDNA.) already contained in a 1.5ml microcentrifuge tube **by slowly**

adding them to the DNA, while stirring with the pipette tip at the same time (use of wide-bore tip is recommended). Make a few transformations in parallel.

17. Incubate the spheroplasts/DNA mixture 10 min at room temperature.
18. Add **0.8ml 20% PEG solution**, and mix by inverting the tube 10 times.
19. Incubate the tube **20 min** at room temperature.
20. Microcentrifuge the cells 10 min at **700×g, 4℃**.
21. Remove the supernatant with a 1-ml pipette.
22. Add 800 µl SOS solution, and **resuspend the cells by pipetting up and down with a wide-bore 1-ml pipette tip**.
23. Incubate the tube for 30-40 min at 30℃.
24. Add cell suspension into 8 ml melted top agar prepared in 15ml tube (keep at 60℃), and invert a few times to mix. Immediately pour cells onto selective bottom agar plate containing 5-FOA.
25. Incubate the plates for 4-7 days at 30℃.

Use circular capture vector (~0.1µg) as a positive control. It should give you zero colony. Any pCAP captured construct could be use as control to quality the spheroplast cells. Cells harboring a pCAP-gene cluster vector can form visible colonies in 2-3 days, but cells carrying a recombined vector grow more slowly.

Alternative transformation protocol (time saver version)

Day 1

1. Inoculate single colony of *S. cerevisiae* strain VL6-48N freshly grown on YPD agar supplemented with adenine (100mg/L) into 50ml YPD plus adenine liquid media and grow at 30 ℃ with shaking until OD600 of 0.7-1.0 is reached (12-16h).

Day 2

2. Put the flasks on ice for 10min and then centrifuge the cells in a 50-ml centrifuge tubes for 3 min at 1800×g, 4℃.
3. Remove the supernatant, and resuspend the cell pellets in 50 ml sterile water (4℃) by vortexing. Wash the cells by centrifuging 3 min at 1800×g, 4℃.
4. Remove the supernatant, and resuspend the cells in 50 ml of 1 M sorbitol (4℃) by vortexing. Leave the cells on ice (or 4℃) for 4h to osmotically stabilize the yeast cells prior to forming spheroplasts.
5. Follow the protocol #6 to #25 described above.

Isolation of candidate colonies

During 4-7 days incubation, yeast transformants form visible colonies on the selective agar plate with 5-FOA. Normally, you will get 10-50 colonies for one transformation. To identify desired clones, you need to screen by colony PCR.

1. Pick up colonies from 2-3 plates with toothpicks and transfer them onto new selective agar plates without 5-FOA.
2. Incubate the plate for 2 days at 30℃.
3. Make three replica plates by using filter paper.
4. Incubate the plates for 1-2 days at 30℃.

Colony PCR screening

Multiplex PCR amplifying several different regions in the cloned cluster at the same time is not recommended.

1. Scoop cells from one plate with loop or 200ul pipette tip, and resuspend them into 200ul of solution 1 (10% sucrose, 50mM Tris-HCl (pH8.0), 10mM EDTA) containing 2-ME (1/1000 dilution) and 0.2mg/ml zymolyase 20T (in 200ul PCR tube).

2. Incubate and shake for 2h at 37℃.

Method 1

3. Boil for 5min (thermal cycler can be use, 98℃/5min).

4. Use 0.2ul as a template for 20ul scale PCR reactions.

Clones showing all desired PCR signals are chosen for DNA extraction.

Method 2 (reliable, but more complicated)

3. The suspension was mixed with 400 µL solution 2 (NaOH 0.2M and 1% SDS (w/v)) by inversion.

4. 300 µL solution 3 (KAc, 3M, pH8.0) was added and centrifuge for 10min at maximal speed.

5. The DNA was precipitated by addition of 1 volume isopropanol and centrifugation (maximal speed, 5 min).

6. Dissolve the DNA pellet by 200 µL 10 Mm pH8.0 Tris-HCl.

7. Use 0.5ul as a template for 50ul scale PCR reactions.

DNA extraction from PCR positive yeast clones

1. Pick up identified clone 5ml yeast selective liquid medium (without Trp). (10*N-base stock with 2% glucose, 0.22um filtration)

2. Incubate 20-30h at 30℃ with shaking.

3. Resuspend cells in 200ul of solution 1 containing 2-ME (1/500 dilution) and 0.5mg/ml zymolyase.

4. Incubate and shake for 2h at 37℃.

5. The suspension was mixed with 400 µL solution 2 (NaOH 0.2M and 1% SDS (w/v)) by inversion.

6. 300 µL solution 3 was added and centrifuge for 10min at maximal speed.

7. Remove residual proteins by phenol-chloroform denaturation, and then precipitate and clean up DNA by using IPA and 70% EtOH.

8. Dissolve DNA in 50ul of 10 Mm pH8.0 Tris-HCl , and use the DNA solution for *E. coli* transformation.

Maintenance of the construct in *E.coli*

The capture vector has the *aph* (Neomycin / Kanamycin resistance) gene on its backbone. Use 50ug/ml Kanamycin for the selection in both *E.coli* and *Streptomyces* expression host.

As described earlier, pCAP03 has pUC-ori for maintenance in *E.coli*. pUC-ori is a high-copy number rep origin (100-500 / cell) derived from pBR322 low-copy rep origin (~20 / cell). Therefore, enough amount of pCAP-derived construct can be easily prepared from conventional *E.coli* cloning hosts.

From the point of view of plasmid stability, low-copy replication would be better in general. However, SuperCos1 utilizing pUC-ori can carry relatively large DNA fragment (~ 60KB) very stably. Likewise, it has been confirmed that pCAP capture vector equipped with pUC-ori from SperCos1 can also carry huge DNA fragment (at least up to 75KB). If your construct has insert larger than 75KB, some stability issues may happen in maintenance in *E.coli*. For propagation of large construct, use of *recA*- and *endA*- strain such as *E.coli* Top10 or *E.coli* strain Stbl4 (invitrogen, Genotype: *mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1 *gyr*A96 *gal*- *thi*-1 *sup*E44 λ- *rel*A1 Δ(*lac*-*pro*AB)/F' *pro*AB+ *lac*IqZΔM15 *Tn*10 (Tet^R)) is recommended to avoid unwanted rearrangement of the cloned cluster. In the $recA^+$ and/or $endA^+$ *E.coli* strains such as BW25113 (for λ-red recombination) and ET12567 (used for conjugal DNA transfer), this stability issue tends to arise. If you encounter this, in order to increase its stability, copy number of your construct can be easily lowered (to original pBR322 level) by cultivating *E.coli* host cells at 30℃, since pUC-ori shows high copy number replication only at higher temperature than 30℃ (for example, at 42℃, it shows extremely high copy replication). By this copy number control strategy, relatively large constructs can be maintained stably in *E.coli*.

DNA extraction from *E.coli* cells

If the size of cloned cluster is smaller than 40KB, Qiagen plasmid extraction kit should work. Use warmed up 0.2x TE for elution. For extraction of huge construct, use traditional mini-prep method. Then check restriction patterns to confirm direct cloning of the gene cluster.

Part 2. Heterologous Expression of Captured Biosynthetic Gene Cluster in *Streptomyces* strains

Intergeneric triparental conjugation for introduction of DNA in *Streptomyces*

The pCAP01 and pCAP03 captured gene cluster can be directly used for conjugation, since these vectors are all contain *oriT* on the vector backbone. For If the Streptomyces host for heterologous expression carries a methyl-sensing restriction system (as is the case for *S. coelicolor* and *S. avermitilis*), it is necessary to passage the pCAP-gene cluster through a non-methylating *E. coli* host such as *E. coli* ET12567. If the target *Streptomyces* does not carry a methyl-sensing restriction system (as is the case for *S. lividans*), common E. coli strains such as DH5α can be used instead. Anyway, *E. coli* ET12567 is suitable for all types of conjugation.

1. Prepare competent cells of E. coli ET12567 grown at 37ºC in LB containing chloramphenicol (25 μg/ml) to maintain selection for the dam mutation.

2. Transform competent cells with pCAP-gene cluster, and select for the incoming plasmid using kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml).

3. Inoculate an *E. coli* ET/pCAP-gene cluster colony and E. coli ET12567/pUB307 into 10 ml LB (chloramphenicol, 25 μg/ml and kanamycin, 50 μg/ml). Grow overnight at 37ºC.

4. Inoculate 100 μl overnight culture into 10 ml fresh LB plus antibiotics as above and grow for about 4 h at 37°C to an OD600 of 0.4-0.6. (Normally, E. coli ET12567/pUB307 should grow faster than *E. coli* ET/pCAP-gene cluster)

5. Wash the cells twice with 10 ml of LB to remove antibiotics that might inhibit *Streptomyces*, and resuspend *E. coli* ET12567/pUB307 in 0.5-1 ml of LB and *E. coli* ET/pCAP-gene cluster in 0.2-0.4 ml of LB, respectively.

6. While washing the E. coli cells, for each conjugation add 10-20 μl *Streptomyces* spores to 200 μl 2 × YT broth. Heat shock at 50°C for 10 min, then allow to cool.

7. Mix 0.1 ml of each *E*. *coli* cell suspension and 0.2 ml heat-shocked spores.

8. Plate out 50 µl and 350 µl of mixture on two MS agar + 10mM MgCl2 (without antibiotics) and incubate at 30°C for 16-20 h.

9. Overlay the plate with 1.5 ml water containing 0.5 mg nalidixic acid (20 µl of 25 mg/ml stock; selectively kills *E*. *coli*) and 2-4 mg kanamycin (40 µl of 50 mg/ml stock). Use a spreader to lightly distribute the antibiotic solution evenly. Continue incubation at 30°C.

10. Pick up a single colony and strike on a MS agar plate containing nalidixic acid (25 µg/ml) kanamycin (50 µg/ml) for single colony and get rid of *E*. *coli* contamination.

11. Preparation of spore suspension for 2-3 independent colony on the MS plate.

Preparation of spore suspensions of *Streptomyces*

To prepare spore suspensions, *Streptomyces* strains were spread on MS agar and incubated at 30 °C for about a week.

The plates were grown till they were well sporulated. 4 mL of sterile ddH_2O were added to each plate and the spores scraped off of the top of the plates and into suspension. The resulting spore suspension was poured into a falcon tube and vortexed vigorously (about 1 min). The spores were separated from the mycelium by passing the suspension through sterile cotton plugged in a disposable syringe. Spores were collected by centrifugation (2,100×g, 10 min, 4 °C), and resuspended in 0.5-2 mL of 20% glycerol. The spore suspensions were stored at -80°C.

Cultivation of *Streptomyces*

Streptomyces strains were routinely cultured in liquid or solid TSB medium. Liquid cultures were carried out in baffled Erlenmeyer flasks containing a stainless steel spring at 200-220 rpm and 28-30 °C for 2 to 3 days. For preparation of protoplasts, *Streptomyces* were cultured in YEME medium containing 0.5% glycine; For isolation of genomic DNA, *Streptomyces* were cultured in YEME or TSB medium. An appropriate concentration of antibiotic was added, if required.

Production of secondary metabolites

For the expression of captured gene cluster thereof, 1 mL of a two-day-old TSB culture of the respective *Streptomyces* strain was inoculated into 50 mL certain medium and grown at 30 °C and 220 rpm for 7 days.