E. coli Expression Vector for Bromoalterochromide Gene Cluster

Lauren Gulland, Avena Ross, Peter Jordan, and Bradley Moore

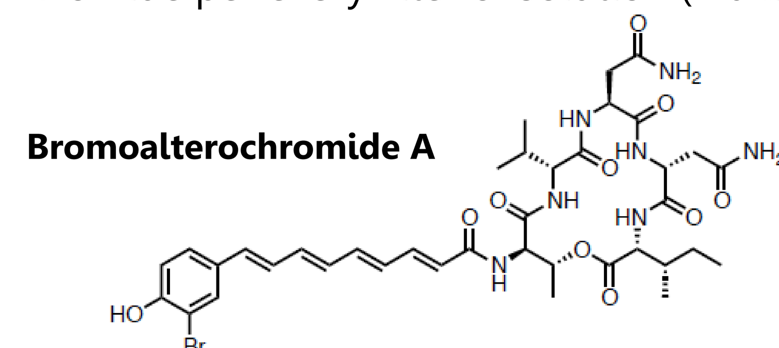
Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, 9500 Gilman Drive, La Jolla, CA

Overview

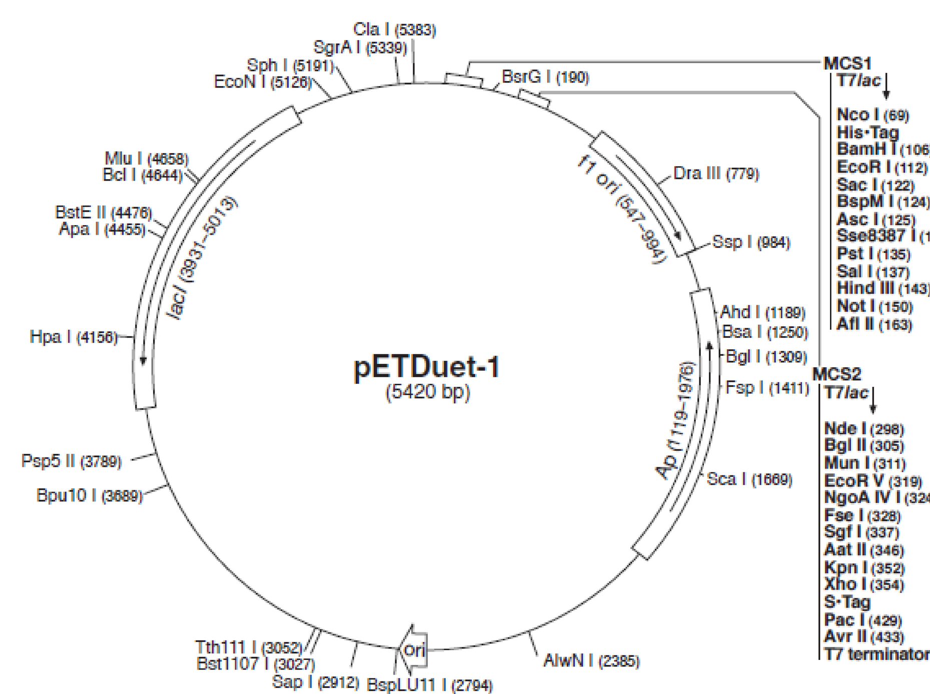
The main point of our project was to confirm the proposed gene cluster responsible for producing Bromoalterochromide A and see if it could be placed into an expression plasmid and be able to be made by *E. Coli*.

Background

Bromoalterochromide A is a compound naturally produced by the marine bacteria *Pseudoalteromonas piscicida* JCM 20779, and has been captured on the pCC01 plasmid from genomic DNA using Transformation Associated Recombination. Since bromoalterochromide is highly brominated and is produced by a marine bacteria, it must be grown in Marine Broth 2216 (from Bectin Dickinson) with an extra gram of Potassium Bromide per every Liter of solution (making 1.08g/L total KBr).



Our Vector: pETDuet-1



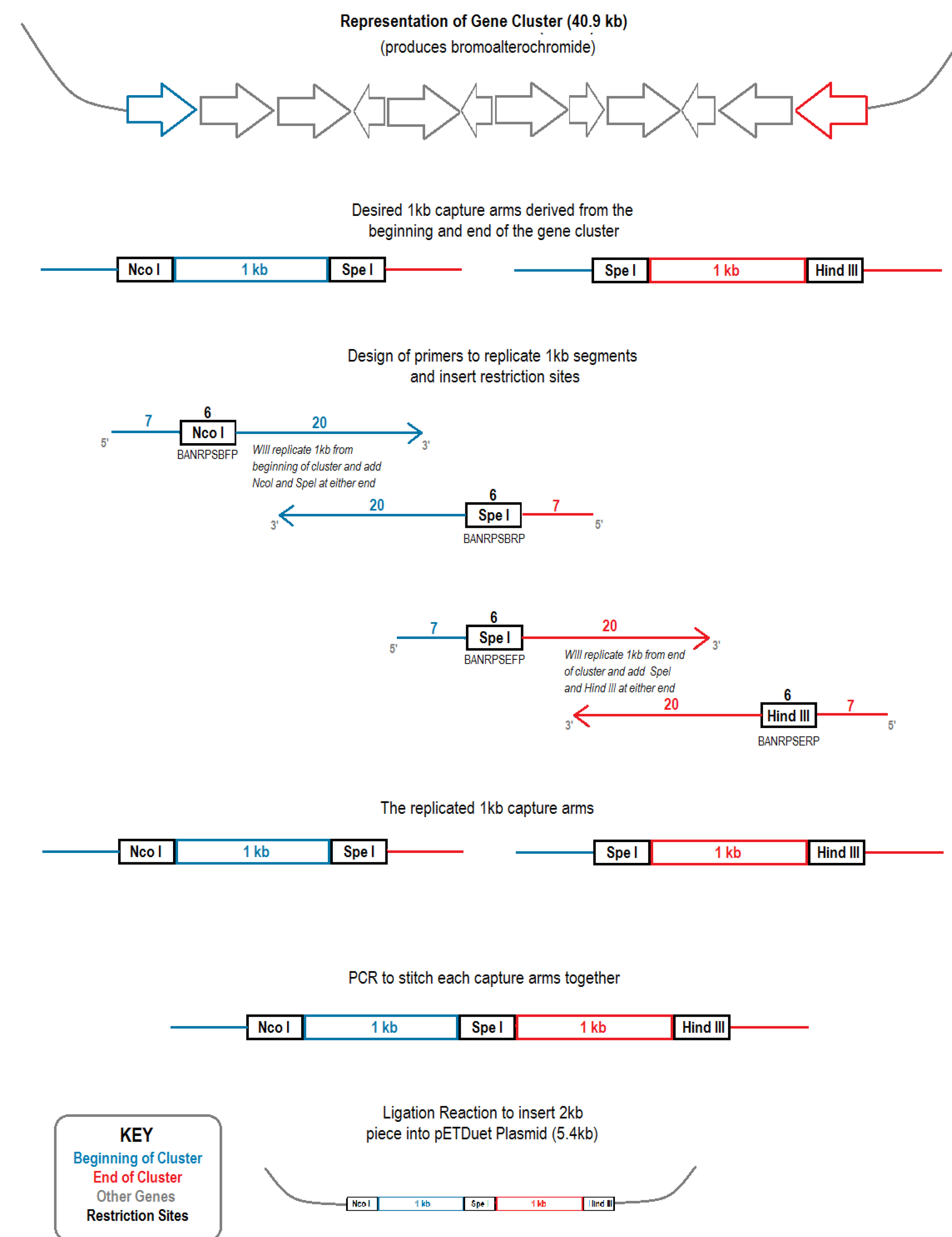
We decided to use pETDuet-1 as our plasmid into which we would insert the desired gene cluster. This specific plasmid has two Multiple Cloning Sites (MCS), into which we place our genes. We were using a version of the plasmid that already had the pptase 2ta16 gene in MCS2, courtesy of Bimo El Gamal, and were attempting to insert our bromoalterochromide gene cluster into MCS1.

Primers

Name of Primer	Sequence (Restriction Site is bolded)	Restriction Site Present
BANRPSBFP	ATATATT**CCATGG**ACCAGTACAACACAGAA AAGGAAGCAATTC	NcoI
BANRPSBRP	GACTAACGC**ACTAGT**GTGATGCCCACATCT CAGCATCAAT	SpeI
BANRPSEFP	GGGCATCAC**ACTAGT**GCGTTAGTCGCACG CAATTACC	SpeI
BANRPSERP	CGCACGC**AAGCTT**CACGCCATTTCAGGGT TGTTAT	HindIII

We designed our primers started with random unmatched sequences (7 bases), then included a restriction site (6 bases), and then matched the sequence from the gene cluster (20 bases). The primers were placed 1 kb apart on each end in opposite directions to amplify a 1kb segment of the genome (see diagram above).

Assembly of Capture Vector



Our main goal of using this was to create a 2kb capture vector that would be inserted into our plasmid, which we would then open up using the SpeI restriction site. The linearized plasmid had 1kb on each end that matched our desired cluster, so the cluster could be inserted via a simple transformation and a colony PCR.

Results

❖Purified bromoalterochromide A from natural producer for further testing and to serve as a standard

❖Assembled 2kb capture arm based on original gene cluster

❖Purified pETDuet-1 pptase plasmid

❖Assembled capture vector from digested 2kb segment and purified plasmid

❖Attempted preliminary lambda red recombination (results pending) to transfer entire 40kb gene cluster

Procedures

Obtaining the bromoalterochromide gene cluster Clones 1-6/R4C6
We did a restriction digestion to verify that all of our clones were correct, and all of them ended up as positive hits.

Growing *P. piscicida* and Isolating Bromoalterochromide
There was a frozen culture of *P. piscicida* in the freezer, and we made two 50mL cultures from it. We incubated these for two days, and used one to isolate the genomic DNA of the bacteria, and the other to do a liquid-liquid extraction of the bromoalterochromide that was secreted. We first used an organic solvent (EtOAc) to isolate the organic part of the culture, and then used the Rotary Evaporator to remove the solvent.

Making competent cells
We created a stock of competent cells which we would then use to do the final transformation with out of BW cells with the pIJ790 and pCC01 BAR4C6 plasmids. The pIJ790 plasmid has Chloramphenical resistance, but when incubated at 37°C, would be ejected from the cell., meaning that the final transformed cells would not contain this plasmid.

Replicating 1kb pieces and sewing into 2kb piece
We ran two separate PCR reactions to replicate each 1kb end of the cluster. For the beginning of the cluster, we used BANRPSBFP and BANRPSBRP (see "Primers" heading), and for the end of the cluster, we used BANRPSEFP and BANRPSERP, which we designed ourselves. For each end, the primers replicated in opposite directions and were spaced 1kb apart in order to only get the desired capture arms. After they were amplified, we purified them and ran another PCR with no primers. Since there is overlap between the two segments (see diagram to the left), the 1kb pieces were essentially stitched together, coming together at the SpeI restriction site. We then ran a PCR with the BANRPSBFP and BANRPSERP primers to replicate the entirety of the 2kb segment.

Restriction Digestions and Ligation Reaction of the 2kb and plasmid
We ran two restriction digestions of the 2kb segment and the pETDuet-1 plasmid at the NcoI and HindIII sites, and incubated the reaction overnight in 37°C incubator. We then gel purified each of the products, and ran a ligation reaction with both of them to insert the 2kb segment into the plasmid. We then purified the reaction and transformed cells with the recombinant plasmid via electroporation. We picked 7 colonies to screen, and two of them had positive hits for the 2kb insert.

Transforming cells with linearized plasmid
We picked one of the two positive colonies to do another restriction digest on at the SpeI site to open up the plasmid with the capture arms and insert the full gene cluster. We then gel purified the plasmid to make sure that we only got the linearized plasmid sample and not any leftover circular plasmids. We then took this and transformed our competent BW pIJ790 pCC01 BAR4C6 cells via electroporation and incubated them overnight at 37°C, which should have ejected the pIJ790 and inserted the gene cluster from the pCC01 into the opened pETDuet-1.

Acknowledgements

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