Appendix I

Clean Deletion of the *nth* Gene from the Chromosome of CC104 *Escherichia Coli*

Introduction

The methods described here are adapted from those published previously [1]. Briefly, overlap extension PCR is used to create a construct in which regions upstream and downstream of a gene of interest (*nth*) are amplified and fused together, devoid of the *nth* gene. This construct is then cloned into the plasmid pKOV [1], which is then transformed into the recipient strain, CC104. During this transformation reaction, the pKOV plasmid can integrate itself onto the CC104 chromosome by homologous recombination. A second recombination event then permits the plasmid backbone to excise itself from the chromosome, leaving only the construct that is devoid of *nth* gene on the chromosome.

Materials

All strains and plasmids were obtained from the Coli Genetic Stock Center at Yale University (New Haven, CT). All enzymes were obtained from New England Biolabs and primers were ordered from Integrated DNA Technologies.

Preparation of *nth* Deletion Construct by Overlap Extension PCR

In order to prepare the Δ*nth* construct, regions upstream and downstream of the *nth* gene were PCR amplified and these products were used as templates in a second PCR experiment, resulting in a product of ~ 2000 bp that omits the *nth* gene (Figure I-1). This product was ligated into the BamHI and NotI sites of pKOV.

The pKOV vector is temperature sensitive, encodes chloramphenicol resistance, and contains the *sacB* gene, which confers sensitivity to sucrose [2].

Plasmid Integration by Homologous Recombination

Electrocompetent CC104 cells were transformed with the pKOV plasmid containing the Δ*nth* construct and then recovered at 30°C. The temperature sensitivity of pKOV necessitates this low recovery temperature. During recovery, the plasmid has the opportunity to recombine onto the CC104 chromosome as shown in Figure I-2.

After recovery, the transformation reactions were plated onto Luria-Bertani (LB) + chloramphenicol medium and incubated at either 30°C or 42°C. Colonies incubated at 42°C are unable to replicate the temperature-sensitive pKOV plasmid and will only maintain their chloramphenicol resistance if this plasmid is integrated onto the chromosome. After incubation, the ratio of colony number on the 42°C plate to colony number on the 30°C plate can be used to calculate the integration efficiency.

Excision of pKOV from the Genome of CC104 to Create a Δ*nth* Strain

Integration of the pKOV plasmid onto the CC104 chromosome leaves the cells with two copies of the upstream and downstream *nth* regions, one from the WT cell and one from the pKOV plasmid. To create the CC104 Δ*nth* strain, one of these copies of the region must be excised. This excision process is also

recombination-based, as diagrammed in Figure I-3. Cells that have excised the pKOV plasmid backbone can be selected based on their tolerance of sucrose. The *sacB* gene of the pKOV plasmid encodes sensitivity to sucrose. If a cell has excised the plasmid backbone from its chromsome, then the cell should be able to grow in the presence of 5% sucrose. If the cell has not excised the pKOV backbone, then sucrose is lethal to the cell. The sucrose selection was conducted by taking cells that had been incubated at 42°C in the previous step, growing them in liquid culture, and then serially diluting these cultures and plating them onto LB + sucrose medium.

Colony PCR to Confirm the Absence of the *nth* Gene

Cells that are able to grow in the presence of sucrose have excised the pKOV plasmid from their chromosome, but they still may not be Δ*nth* strain. As Figure I-3 shows, the orientation of the chromosomal DNA during recombination may create a Δ*nth* strain, or it may cause the cell to revert to WT. Consequently, a final screen is needed to determine which of the sucrose-tolerant cells are truly Δ*nth* mutants. To verify that the *nth* gene has been deleted, PCR was used with primers that bind upstream and downstream of the region containing the *nth* gene. The size of the PCR product indicated whether the colony examined was WT or Δ*nth*. In this reaction, a ~ 2000 bp PCR product was expected of Δ*nth* cells, whereas a ~ 2600 bp product was expected of WT cells (Figure I-4). Of several colonies

examined, three appeared to have the Δ*nth* genotype when examined by colony PCR (Figure I-4).

After this PCR reaction, the samples that appeared to be Δ*nth* were isolated, re-streaked, and analyzed by colony PCR again. This process was repeated at least three times to ensure that the samples were not contaminated with WT CC104. Finally, sequencing was used to verify that the isolated strains were truly Δ*nth*.

References:

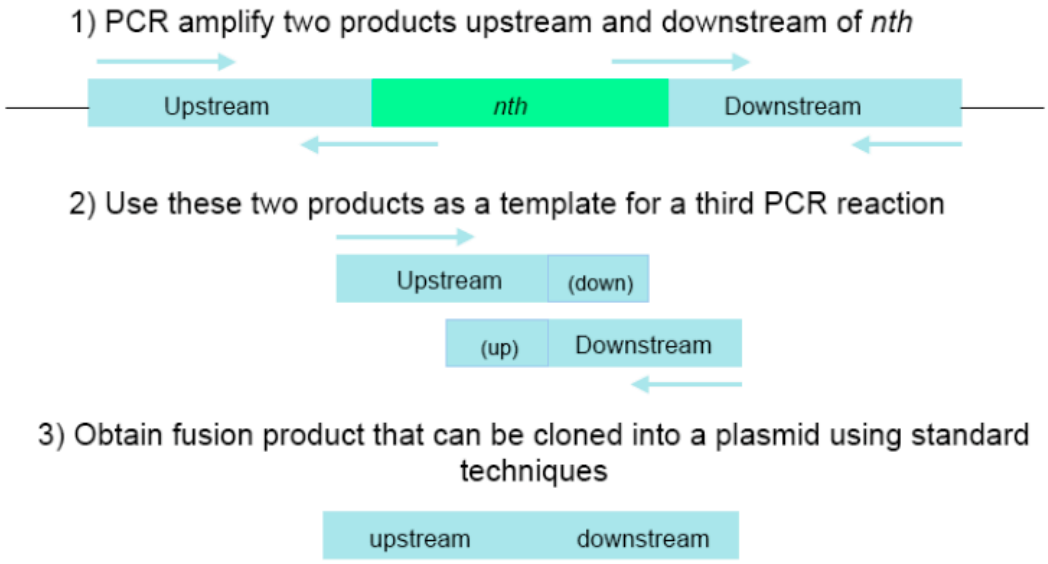
1. Link, A.J., D. Phillips, and G.M. Church, *Methods for Generating Precise Deletions and Insertions in the Genome of Wild-Type Escherichia coli: Application to Open Reading Frame Characterization.* Journal of Bacteriology, 1997. **179**(20): 6228–6237.
2. Dedonder, R., *Levansucrase from Bacillus subtilis.* Methods in Enzymology, 1966. **8**(C): 500–505.

Table I-1: Primers used in this protocol

Name	Sequence	Purpose
CARJKB172	5'-actagtgcggccgcgaactgcgcctttctgactggatc-3'	Primer for overlap extension to create Δ*nth* construct, and for verifying absence of *nth* on CC104 chromosome. It binds to a sequence 1000 bp upstream of *nth* gene.
CARJKB173	5'-ggcgcgccggatcccataaccaatcgccagcacaatagc-3'	Primer for overlap extension to create Δ*nth* construct, and for verifying absence of *nth* on CC104 chromosome. It binds to a sequence 1000 bp downstream of *nth* gene.
delnthfusfor	5'- gcattgccaaacggtgaaacagggaatgtctgatgaagaaaaggggtaacaccgattaccccattg-3'	Primer for overlap extension to create Δ*nth* construct. It binds to a sequence immediately following the *nth* gene.
delnthfusrev	5'-caatggggtaatcggtgttacccctttttcttcatcagacattccctgtttcaccgtttggcaatgc-3'	Primer for overlap extension to create Δ*nth* construct. It binds to a sequence immediately preceding the *nth* gene.
CARJKB174	5'-acattgtttgacggtgcaga-3'	Sequencing Δ*nth* strains
CARJKB175	5'-ggtcaatatttgttgcgtgtg-3'	Sequencing Δ*nth* strains
CARJKB180	5'-gcaatggcacattgtttgac-3'	Sequencing Δ*nth* strains

Figure I-1: Experimental schemes for clean deletion construct preparation. A) Schematic of how overlap extension PCR can be used to create a plasmid insert that contains a section of the CC104 chromosome devoid of *nth*. B) PCR results indication that regions 1000 bp upstream and downstream of the *nth* gene were successfully amplified and fused together through PCR.

A)



B)

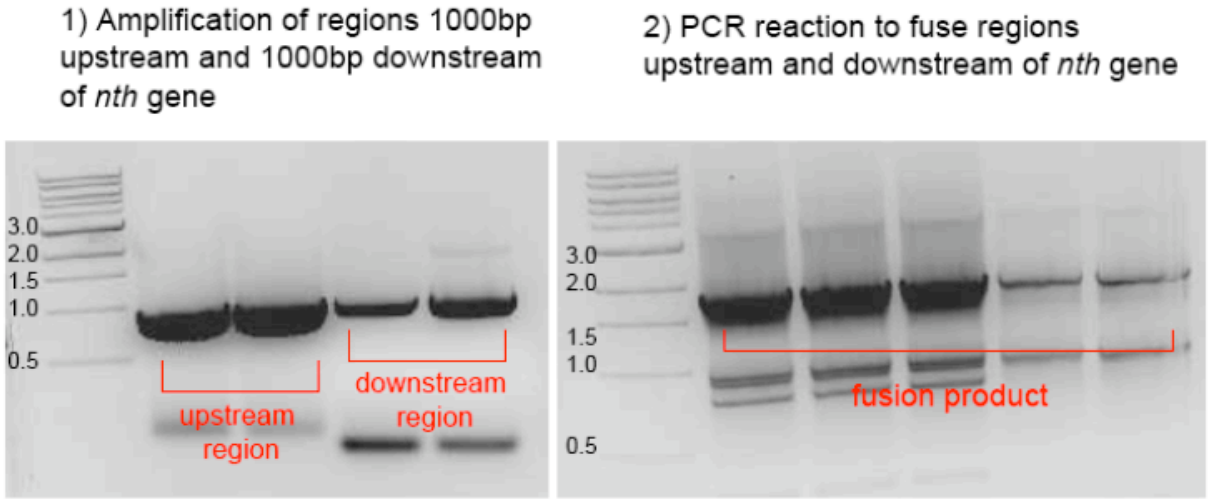


Figure I-2: Diagram of how pKOV can integrate onto the chromosome of CC104

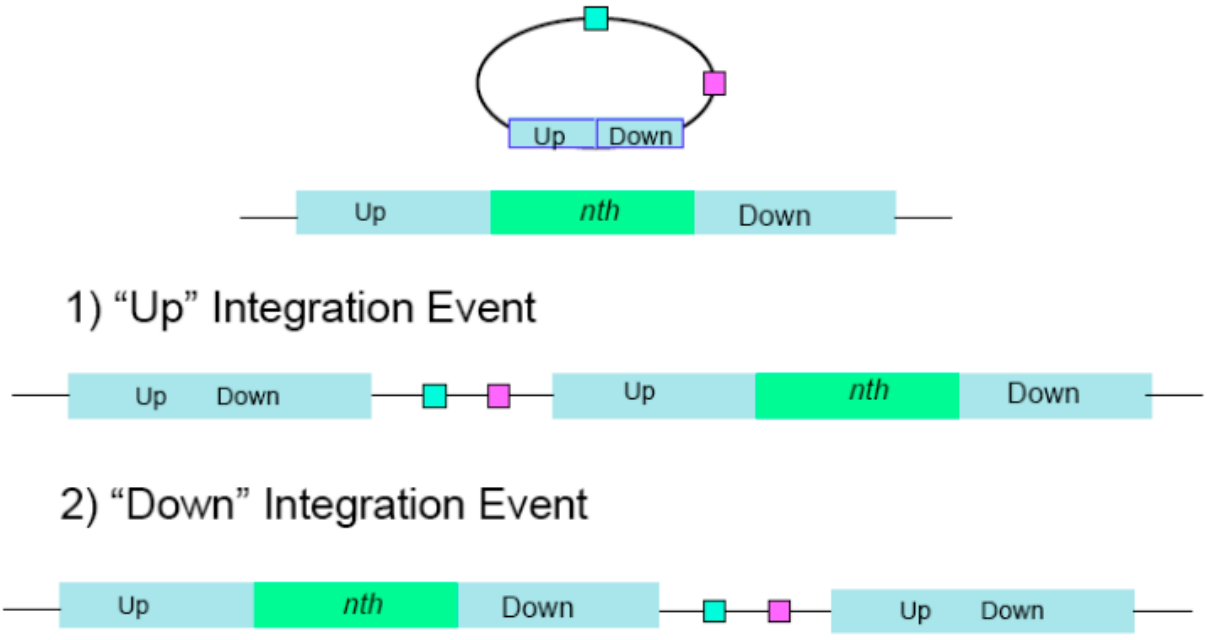


Figure I-3: Scheme for recombination-based excision of the pKOV plasmid. The plasmid should excise from the chromosome of CC104, yielding a single copy of the chromosomal region of interest. Note that several different products are available after this recombination/excision event, and further screens are necessary to select the strain of interest.

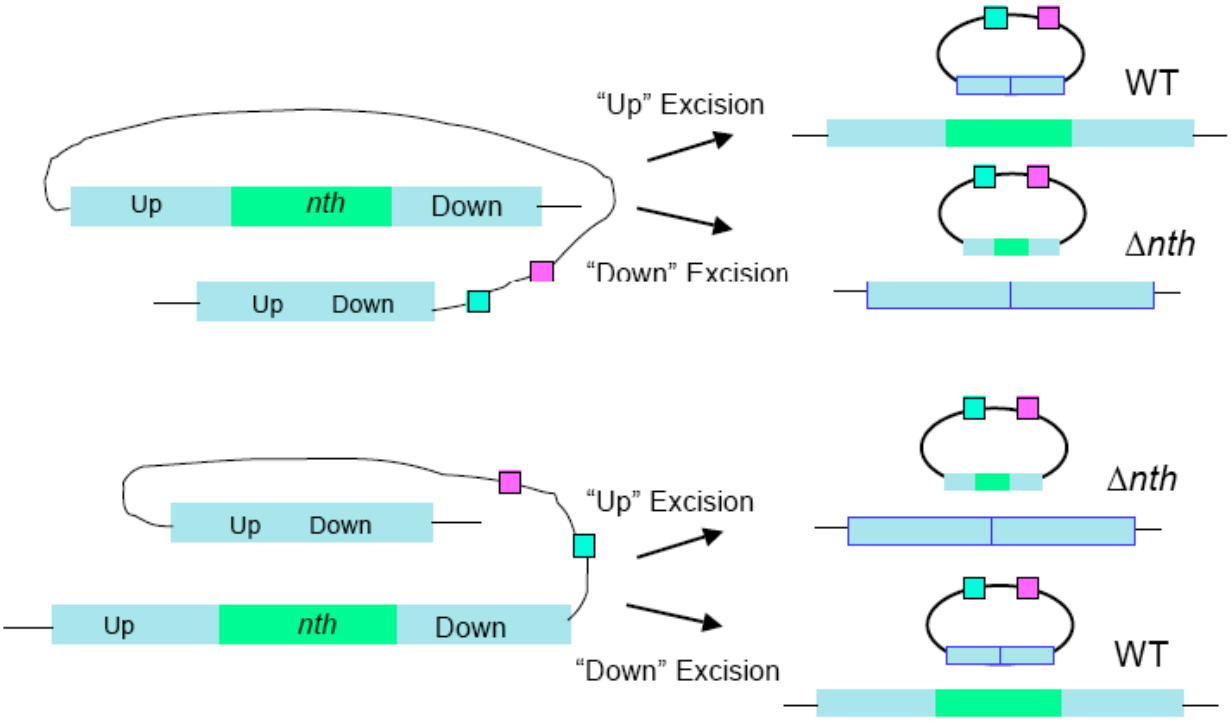
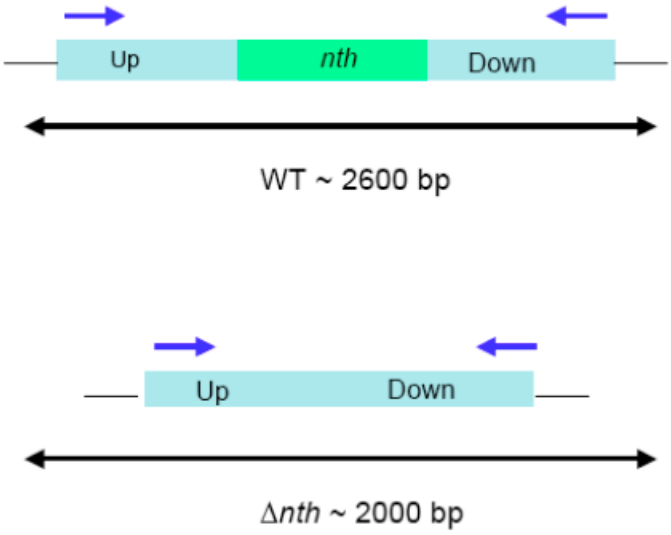


Figure I-4: Schematic for verification of clean deletion construct. A) Scheme of how colony PCR can be used to determine the presence or absence of the *nth* gene on the chromosome of CC104. B) Results of the screen of several colonies that detected three samples (colonies 19, 22, 23) that are possibly Δ*nth*. The lane labeled "WT" was a control performed with WT CC104, and the lane labeled "pKOV" was a control performed with the pKOV vector containing the Δ*nth* construct. The lane labeled "blank" was a cell-free control, and all other lanes are results using colonies taken from LB-sucrose plates.

A)



B)

Appendix II

Insertion of the *nth* Gene onto the Chromosome of *Escherichia Coli* at the λ Phage Attachment Site

Introduction

The methods described here are adapted from those previously published [1]. Briefly, EndoIII mutants of interested were cloned into a plasmid, pAH120 [1], which has the ability to integrate itself onto the chromosome of *Escherichia coli* at the λ phage attachment site. When this plasmid integrates, the allele of *nth* that it contains also integrates, thus creating a strain of CC104 that expresses a mutant version of the EndoIII protein.

Materials

All strains and plasmids were obtained from the Coli Genetic Stock Center at Yale University (New Haven, CT). All enzymes were obtained from New England Biolabs and primers were ordered from Integrated DNA Technologies.

Preparation of pAH120 with *nth* Alleles and Chromosomal Integration

EndoIII variants were PCR amplified from overexpression vectors and then ligated into the BamHI and NdeI sites of pAH120. Samples of pAH120 expressing different variants of *nth* were transformed into electrocompetent CC104Δ*nth* cells that were also expressing the plasmid pINT-ts. The latter plasmid is temperature sensitive and encodes integrase genes that help the pAH120 plasmid integrate onto the bacterial chromosome. Following transformation, the cells were incubated at

30ºC for one hour, during which time pAH120 may integrate onto the chromosome, and then at 42ºC for 30 minutes, during which time pINT-ts is cured. Cultures were then spread onto Luria-Bertani (LB) + kanamycin media and incubated at 37ºC. This final incubation selects for cells that have integrated pAH120, since this plasmid confers kanamycin resistance.

Colonies that emerge on these kanamycin selection plates were then screened using colony PCR to verify the presence of the *nth* variant on the chromosome. Colony PCR was performed with primers specific to the *nth* gene. A ~ 600 bp product is expected if the samples contain an *nth* variant. As Figure II-1 demonstrates, all colonies examined appeared to contain a variant of *nth*.

Verification That Only One Plasmid Integration Event Occurred

A further PCR screen is then needed to verify that the pAH120 plasmid integrated only once onto the chromosome of a given sample, and not multiple times. The integration events and PCR screen are diagrammed in Figure II-2. Primers were designed such that the size of a PCR product they produce indicates how many times the pAH120 plasmid integrated. Most of the colonies screened only contain one copy of the plasmid on their chromosome (Figure II-2).

References:

1. Haldimann, A., and B.L. Wanner, *Conditional-Replication, Integration, Excision, and Retrieval Plasmid-Host Systems for Gene Structure–Function Studies of Bacteria.* Journal of Bacteriology, 2001. **183**(21): 6384–6393.

Table II-1: Primers used in these experiments

Name	Sequence	Purpose
CARJKB164	5'-ggcgcgcccatatgaataaagcaaaacgcctggagatc-3'	Primer for cloning *nth* alleles into pAH120, NdeI site
CARJKB165	5'-ggcgcgccggatcctcagatgtcaactttctctttgtattc-3'	Primer for cloning *nth* alleles into pAH120, BamHI site
CARJKB176	5'-cgaattcaggcgctttttag-3'	Sequencing *nth* alleles after chromosomal insertion
CARJKB177	5'- tctgctggaaccactttcagt-3'	Sequencing *nth* alleles after chromosomal insertion
CARJKB178	5'-tgaaagtggttccagcagag-3'	Sequencing *nth* alleles after chromosomal insertion
CARJKB179	5'- gggagtgggacaaaattgaa-3'	Sequencing *nth* alleles after chromosomal insertion

Figure II-1: Results of colony PCR to verify presence of *nth* variants. PCR products were amplified from the chromosome of CC104 Δ*nth* after incubation with pAH120. All samples examined appear to have integrated the plasmid and the *nth* variant of interest.

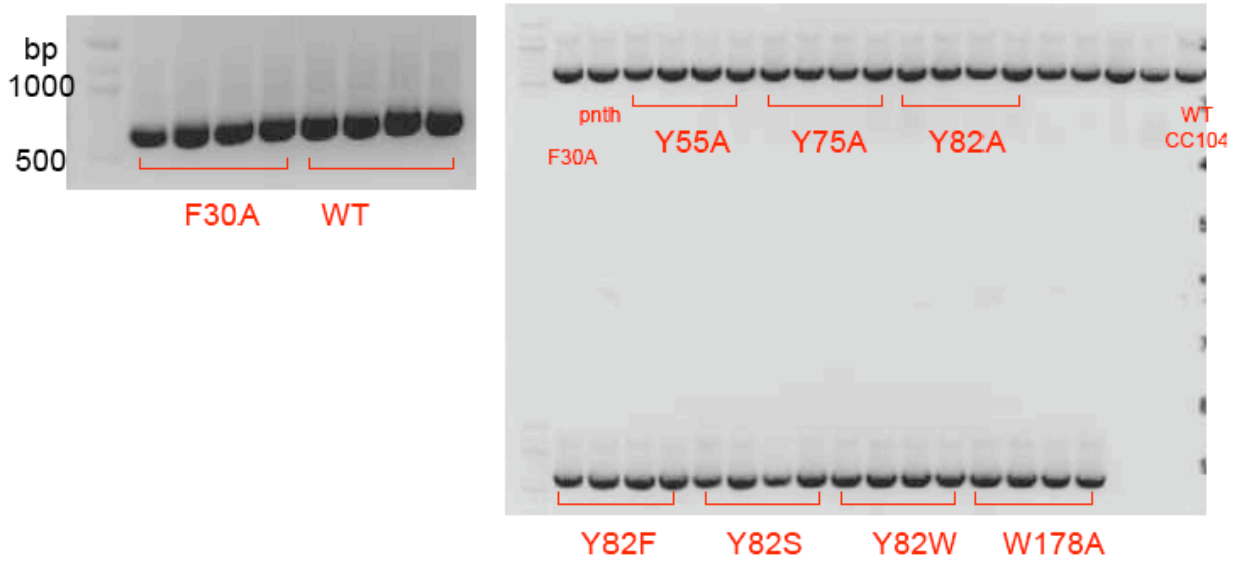
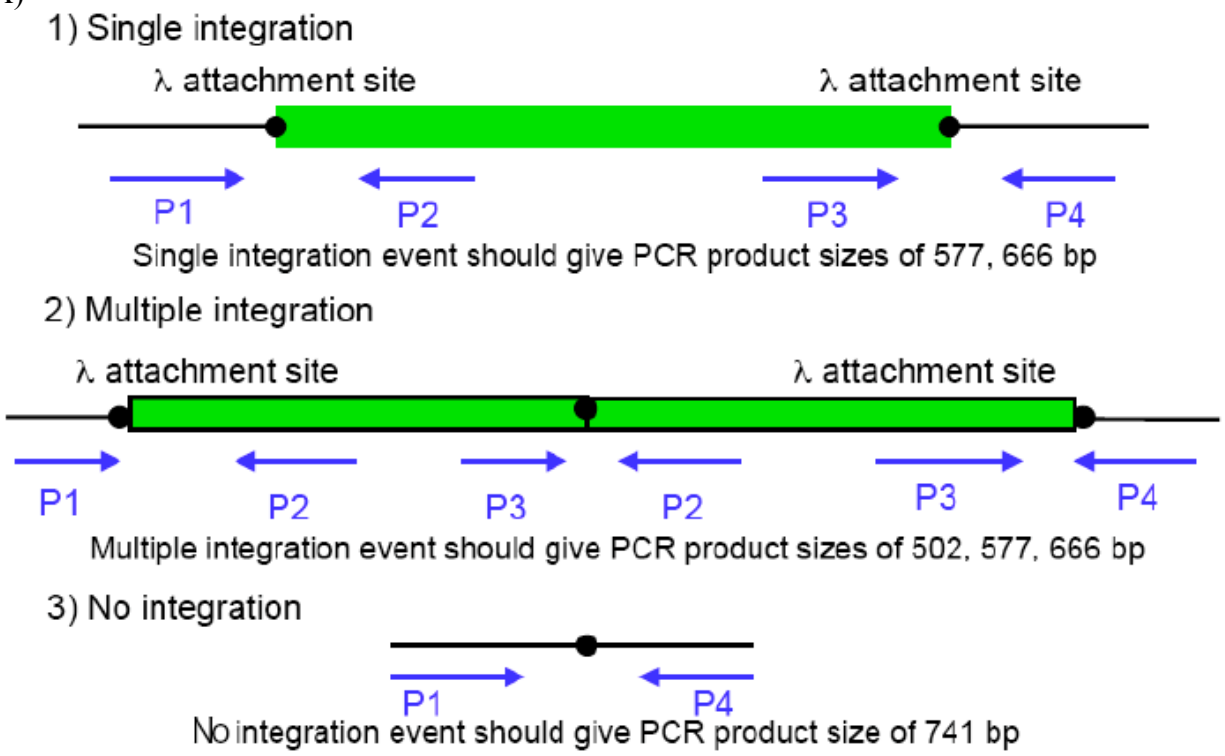
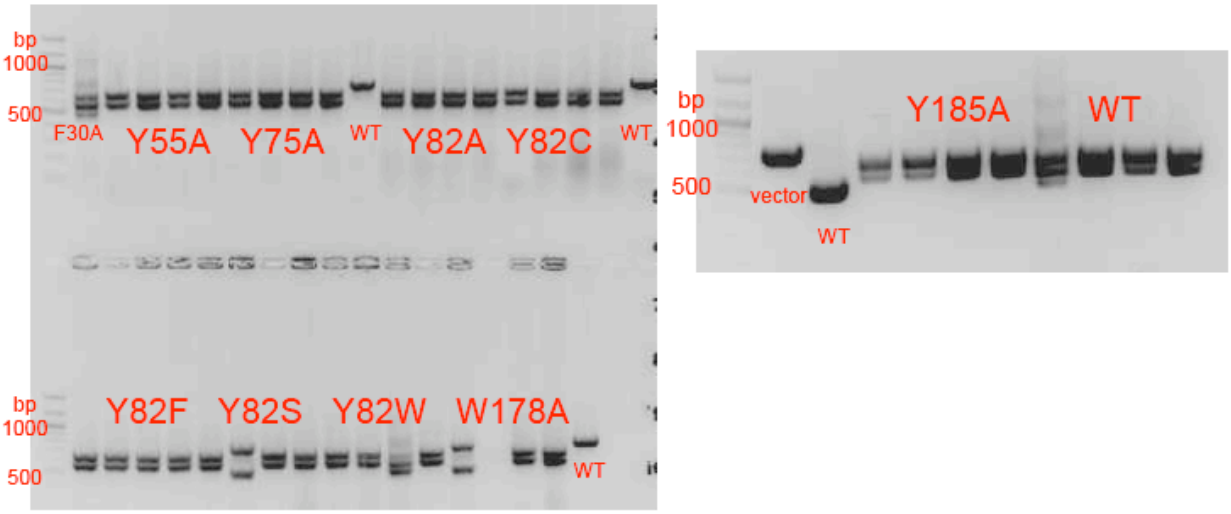


Figure II-2: Diagram of PCR experiments to verify single insertion. A) Diagram of how colony PCR can be used to verify that pAH120 only inserted once and not multiple times. Single integration, multiple integration events, and no integration events will yield products of different sizes. B) Results of colony PCR performed on several samples containing *nth* variants. Most samples display PCR products whose sizes indicate a single integration event.

A)



B)



Appendix III

Insertion of *nth* onto the Chromosome of *Escherichia Coli* Using the Tn7 Transposon

Introduction

The methods described here are adapted from those published previously [1]. The insertion process is also diagrammed in Figure III-1. Briefly, a suicide plasmid containing the Tn7 transposon [2] and the *nth* gene is prepared and then transformed into a donor strain, BW29427. Simultaneously, a separate sample of BW29427 is transformed with the plasmid pUX-BF13, which contains transposase genes that help the Tn7 transposon insert itself onto the *E. coli* chromosome. These two strains of BW29427 are then mated with a recipient, CC104.

During the mating, the two plasmids should enter cells of CC104, and the Tn7 transposon and *nth* gene will be inserted onto the CC104 chromosome. The mating reaction is then re-streaked onto media on which the BW29427 donor cells cannot grow, thus selecting for the CC104 recipient cells. The remaining steps of the protocol describe how to examine these CC104 cells to ensure that they received the Tn7 and *nth* genes, that they have been cured of the donor plasmids, and that the transposition reaction occurred at the correct genomic location.

Materials

The strain CC104 was obtained from Professor Jeffrey Miller at UCLA (Los Angeles, CA), CC104 *nth*$^-$ was obtained from Dr. Amie Boal, BW29427 was obtained from Professor Barry Wanner (Purdue University). The plasmids pURR24 and pUX-BF13 were obtained from Professor Dianne Newman at the

California Institute of Technology. Diaminopimelic acid (DAP), ampicillin, and spectinomycin were obtained from Sigma-Aldrich. Primers were ordered from Integrated DNA Technologies. Enzymes were purchased from New England Biolabs. All media and buffers were prepared according to standard procedures for *E. coli* [3].

Preparation of Donor Strains for Conjugation

The *nth* gene was amplified from a template provided by Dr. Amie Boal (Caltech). The PCR product was ligated into the XmaI site of pURR24 to create pURR24nth. The plasmid pURR24 contains a miniTn7 transposon and it is also a suicide plasmid, meaning it cannot be replicated by many strains of *E. coli*. Consequently, these strains can easily be cured of the plasmid once transposition has taken place.

The plasmid pURR24nth was then transformed into BW29427 *E. coli*. A separate sample of BW29427 was transformed with the plasmid pUX-BF13, which contains the transposase genes that mediate the transposition of Tn7 and *nth* onto the chromosome of an *E. coli* cell. BW29427 cells are unable to make diaminopimelic acid (DAP), a reagent that *E. coli* cells use to maintain the rigidity of their cell walls [4]. The Luria-Bertani (LB) media for BW29427 was supplemented with 300 μM DAP.

Mating Reaction

Overnight cultures of BW29427 with pURR24nth, BW29427 with pUX-BF13, and CC104 *nth*$^-$ were prepared. A 100 μL aliquot of each culture was removed and placed in a sterile microcentrifuge tube. Control experiments were also performed in which only two of the three strains were mixed in the microcentrifuge tube. The tubes were centrifuged at 8000 rpm for two minutes. Their supernatant was decanted, and the cells were resuspended in 50 μL fresh Luria-Bertani (LB) medium, supplemented with DAP. This entire solution was then spread onto an LB + DAP plate and incubated for 3 hours at 37°C.

A sample of cells was collected from each of the plates using a sterile stick and re-suspended in 1 mL fresh LB (without DAP). This solution of cells was serially diluted, and various dilutions were plated onto LB + spectinomycin media and incubated at 37°C. Colonies should only appear if an integration event has occurred because this event would confer spectinomycin resistance. Transconjugant colonies were only observed on plates in which all three strains (BW29427 + pURR24nth, BW29427 + pUX-BF13, and CC104*nth*$^-$) had been mixed (Figure III-2).

Screening of Cells to Verify the Accuracy and Location of Transposition

After the plates have incubated overnight, the colonies that grow need to be examined to ensure that several processes have taken place: 1) The CC104 recipient cells have been cured of any plasmids, 2) Only the *nth* gene and Tn7 transposon

have integrated into CC104, not the entire pURR24 plasmid, 3) The *nth* gene and Tn7 transposon have integrated themselves into the CC104 chromosome, 4) The *nth* gene and Tn7 transposon inserted at the Tn7 attachment site [5, 6] and not elsewhere in the CC104 chromosome, and 5) Only one insertion event occurred, and the recipient cells do not contain multiple copies of the *nth* gene.

To screen for the absence of plasmids in the recipient CC104 *nth*$^-$ cells, a plasmid prep protocol can be performed on several colonies, and the results run on an agarose gel. No plasmids should be visible. When examining cells for the absence of pURR24 plasmid backbone, several screening methods can be applied. Select transconjugants can be re-streaked on LB media containg spectinomycin and 5% sucrose. The pURR24 plasmid backbone contains the *sacB* gene, which encodes sensitivity to sucrose [7]. Therefore, cells that contain pURR24 will not grow on sucrose medium. To enhance the cells' sucrose sensitivity, sodium chloride can be omitted from their growth medium. Simultaneously, transconjugants can be re-streaked onto LB-ampicillin media. Because the pURR24 backbone encodes ampicillin resistance, colonies that have removed it should be ampicillin sensitive. When subjected to these tests, the transconjugants examined displayed sucrose tolerance and ampicillin sensitivity (Figure III-3). PCR with primers specific to the pURR24 backbone further validated the plasmid's absence (Figure III-4).

To ensure that the Tn7 transposon and *nth* gene integrated successfully onto the chromosome of the CC104 recipient, PCR experiments were performed with

primers specific to the transposon-containing region of pURR24. The primers were designed such that the size of the PCR product indicates whether the Tn7 transposon and/or the *nth* gene have integrated onto the chromosome. PCR results indicated that the transconjugants screened did contain the Tn7 transposon and *nth* gene (Figure III-5).

When the Tn7 transposon and *nth* gene insert onto the chromosome of CC104, it is important that their orientation relative to other chromosomal genes be monitored. The screen for proper orientation is also accomplished by PCR (Figure III-5). Primers were designed such that the size of the PCR product indicates whether the gene or transposon is properly aligned on the chromosome. Several transconjugants with the appropriate alignment were obtained. The final screen of the transconjugants will verify that the *nth* gene and Tn7 transposon only inserted in single copy. This screen was not performed for the transconjugants made here, but can be accomplished by sequencing or southern blot.

References:

1. Choi, K.-H., and H.P. Schweizer, *1mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa.* Nature Protocols, 2006. **1**(1): 153–161.
2. Peters, J.E., and N.L. Craig, *Tn7: Smarter that we Thought.* Nature Reviews: Molecular Cell Biology, 2001. **2**: 806–813.
3. Miller, J.H., *Experiments in Molecular Genetics*. 1972, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
4. Baumann, R.J., et al., *Inhibition of Escherichia coli Growth and Diaminopimelic Acid Epimerase by 3-Chlorodiaminopimelic Acid.* Antimicrobial Agents and Chemotherapy, 1988. **32**(8): 1119–1123.
5. Gringauz, E., et al., *Recognition of Escherichia-Coli AttTn7 by Transposon Tn7—Lack of Specific Sequence Requirements at the Point of Tn7 Insertion.* Journal of Bacteriology, 1988. **170**(6): 2832–2840.
6. Mckown, R.L., et al., *Sequence Requirements of Escherichia-Coli AttTn7, a Specific Site of Transposon Tn7 Insertion.* Journal of Bacteriology, 1988. **170**(1): 352–358.
7. Dedonder, R., *Levansucrase from Bacillus subtilis.* Methods in Enzymology, 1966. **8**(C): 500–505.

Table III-1: Primers used in these experiments

Name	Sequence	Purpose
CARJKB6	5'-cccgggtcaatggggtaatcggtgtt- 3'	Cloning of *nth* gene into pURR24, XmaI site
CARJKB8	5'- cgccccggggtggatcctcaatggggtaa-3'	Cloning of *nth* gene into pURR24, XmaI site
CARJKB14	5'-gcgagggctttactaagctg -3'	Verifying presence of *nth* gene in pURR24, and sequencing of this insert
CARJKB15	5'-tccagttatgctgtgaaaaagc-3'	Verifying presence of *nth* gene in pURR24, and sequencing of this insert
CARJKB32	5'-tcgctataatgaccccgaag-3'	Primer is specific to pURR24 backbone, used to verify pURR24 absence in a CC104 sample
CARJKB36	5'-cggtttgtcacatggagttg-3'	Primer matches a region just upstream of Tn7 attachment site, used to verify presence of Tn7 in CC104 sample
CARJKB37	5'-gcaggccaaccagataagtg-3'	Primer matches a region of the Tn7 transposon, used to verify transposon's presence in CC104 sample
CARJKB38	5'-tgctttttcacagcataactgg-3'	Primer matches a region of the Tn7 transposon, used to verify transposon's presence in CC104 sample
CARJKB39	5'- cgataacatgcacatcatcgag-3'	Primer matches a region just downstream of Tn7 attachment site, used to verify presence of Tn7 in CC104 sample
CARJKB42	5'-ttcggtcctccgatcgttgt-3'	Primer is specific to pURR24 backbone, used to verify pURR24 absence in a CC104 sample
CARJKB43	5'-ttctgctatgtggcgcggta-3'	Primer is specific to pURR24 backbone, used to verify pURR24 absence in a CC104 sample
CARJKB44	5'-ttttcacagcataactggactga-3'	Primer matches a region of the Tn7 transposon, used to verify transposon's presence in CC104 sample
CARJKB45	5'-gaagcgctggcagaagattt-3'	Primer matches a region just downstream of Tn7 attachment site, used to verify presence of Tn7 in CC104 sample

Figure III-1: Overview of gene insertion using the Tn7 transposon

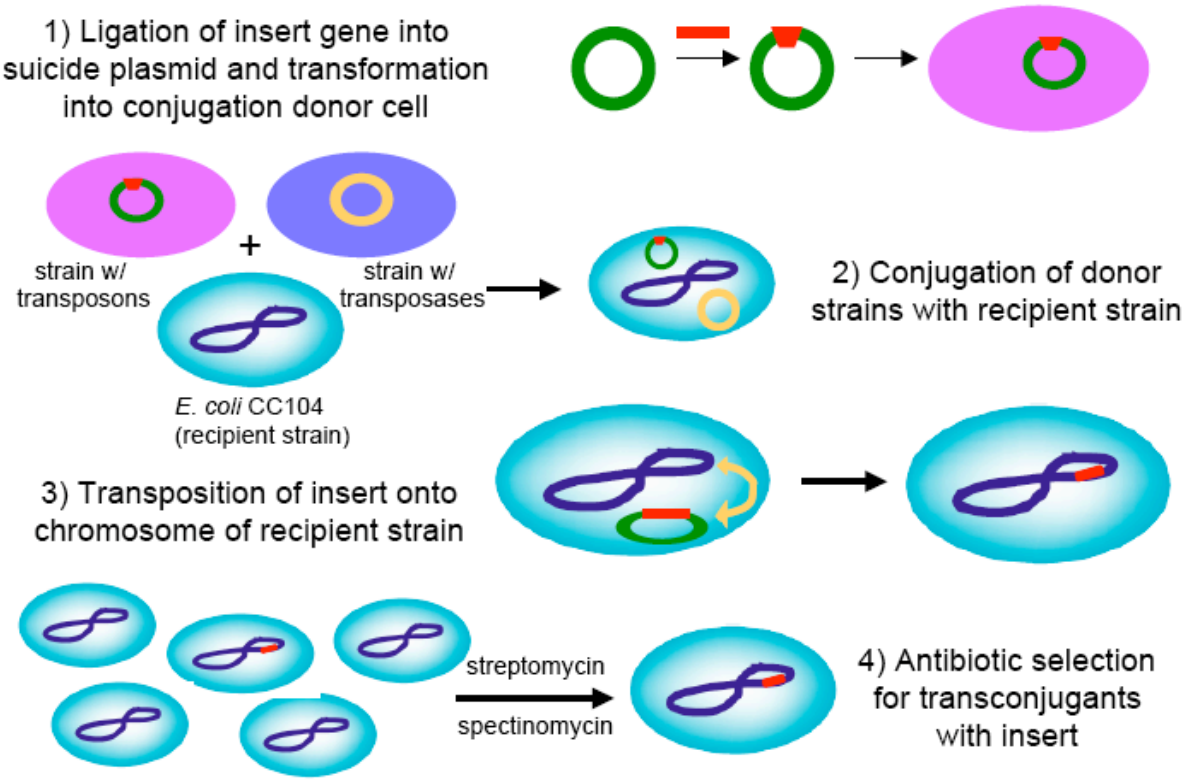


Figure III-2: Results of mating showing that transconjugant colonies form only when all three parent strains are used in the mating reaction

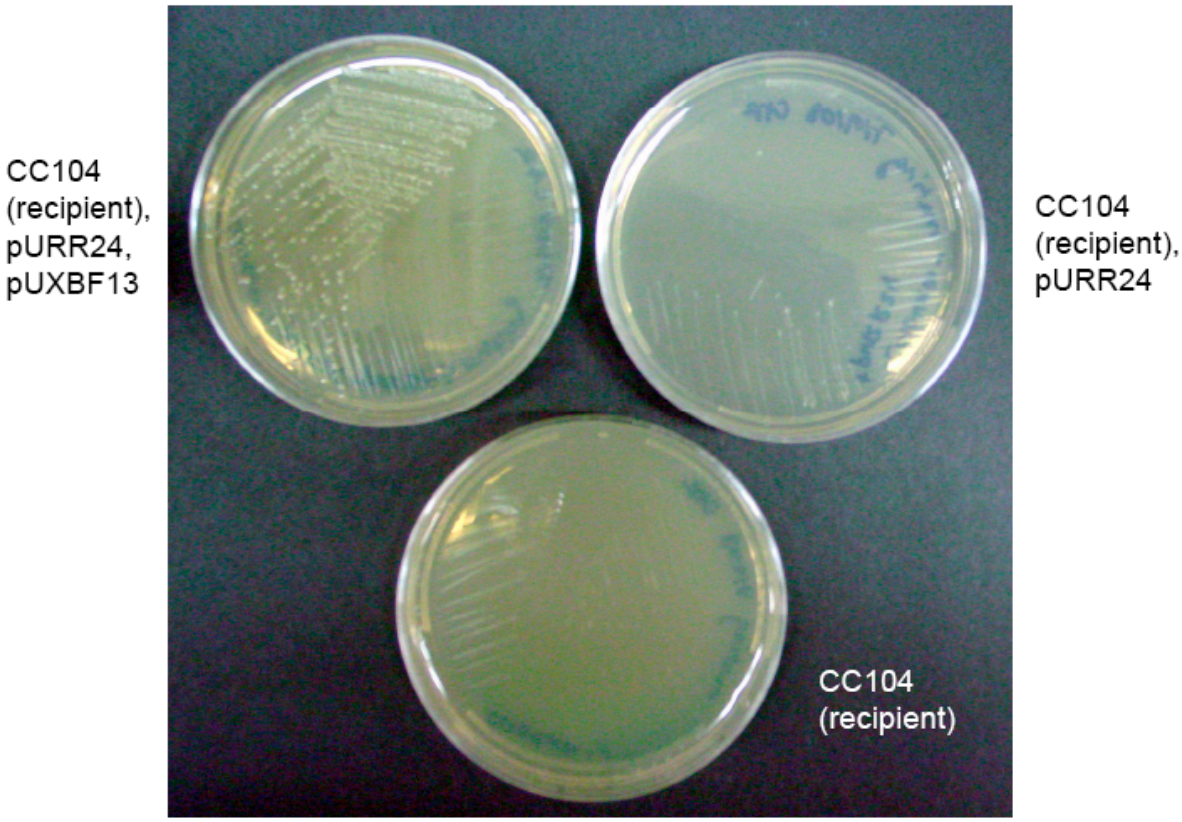


Figure III-3: Transconjugants were re-plated on sucrose-containing and ampicillin-containing media. The results are shown for sample number eight. If the pURR24 backbone is not present on the strain's chromosome, then the strain should be sucrose-tolerant and ampicillin-sensitive.

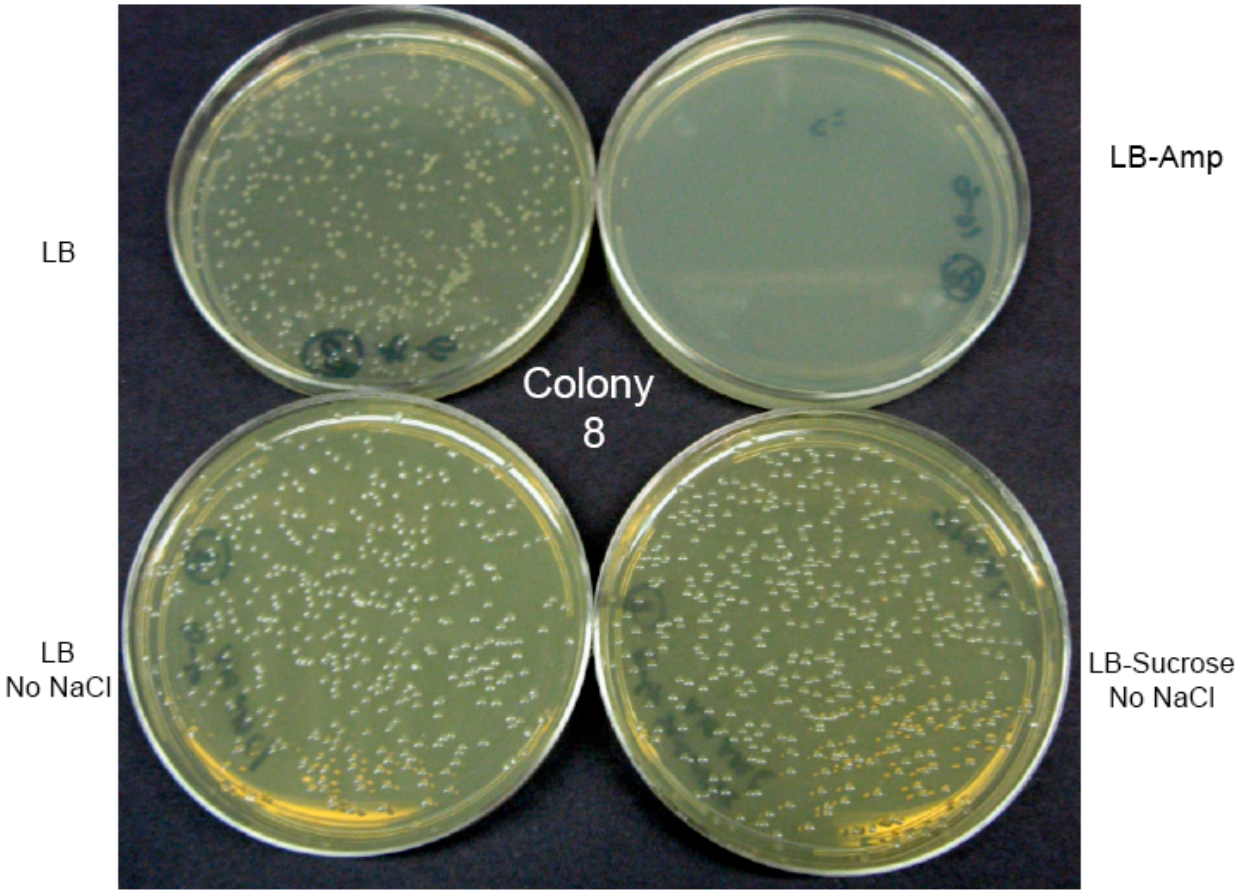


Figure III-4: Results of PCR experiments with primers specific to the backbone of pURR24. Since no product of the appropriate size is detected, this result corroborates those described in Figure III-3 and suggests that the pURR24 backbone is not present in most of the colonies examined.

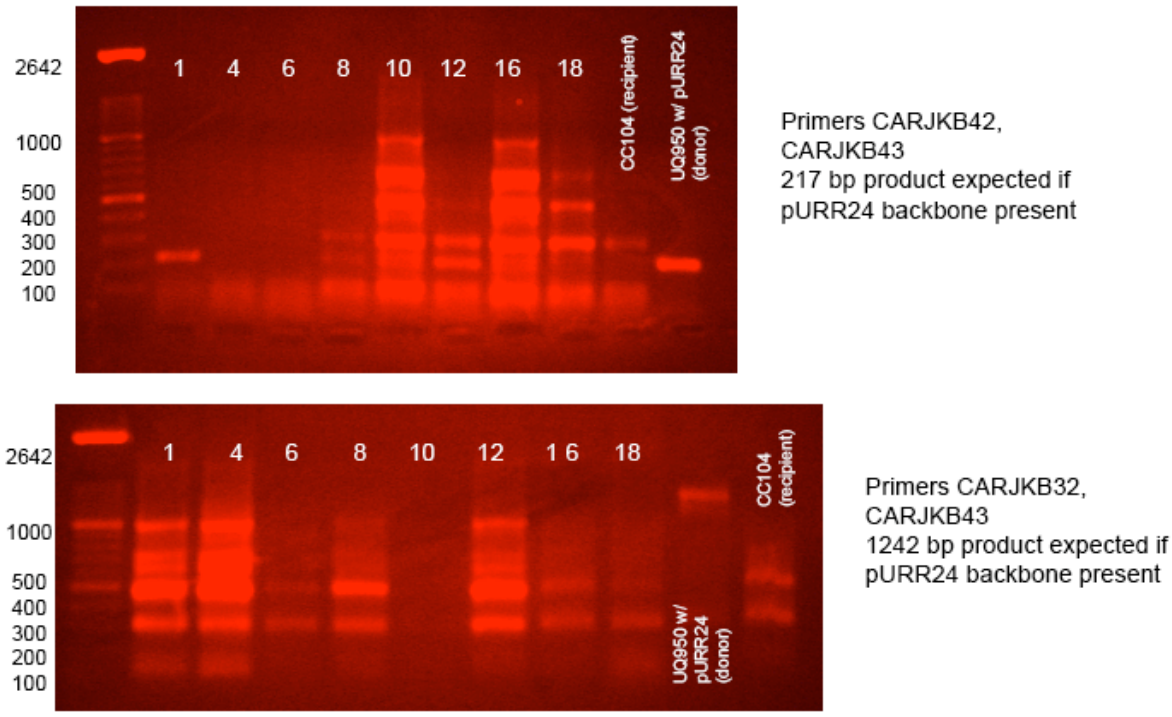


Figure III-5: Results of PCR experiments with primers specific to the Tn7 transposon and *nth* gene. These experiments demonstrate that the *nth* gene and Tn7 transposon are present on the chromosomes of the samples examined, and also that the insert is in the correct orientation relative to the genes that flank the Tn7 attachment site.