APPENDIX II

Inactivation of genes in *Escherichia coli*

INTRODUCTION

The methods described here are adapted from those published previously (*1*).

MATERIALS AND METHODS

Materials

The CC104 strain was obtained from Prof. Jeffrey Miller at UCLA (Los Angeles, CA), CC104 *mutY-* and CC104 *mutY-/mutM-* strains were obtained from Prof. Sheila David at UC Davis (Davis, CA), and the CC102 strain was obtained from the *E. coli* Genetic Stock Center at Yale University (New Haven, CT). pKD3, pKD4, and pKD46 were obtained from Prof. Dianne Newman at MIT (Boston, MA). Enzymes were purchased from New England Biolabs or Stratagene. All media and buffers were prepared according to standard procedures (*2*).

Generation of FRT-flanked Resistance Gene

Figure A2.1 shows the overall experimental strategy developed by Wanner *et al.* for gene inactivation in *E. coli*. Primers containing priming regions for amplification of pKD3 as well as sequences flanking the *nth* or *rnfA* region in *E. coli* were designed and these are shown in Table A2.1. These were purchased HPLC purified from Qiagen (or IDT) and dissolved in 10 mM Tris, pH 7.5 at a final concentration of 100 μM. These were used in a PCR reaction using purified pKD3 as the template where each reaction contained 85 μL sterile water, 10 μL *Pfu* buffer (Stratagene), 2 μL dNTPs, 0.5 μL each primer, 1 μL template, and 1 μL of a 9:1 mixture of *Taq* and *PfuTurbo* polymerases

Figure A2.1. Scheme for gene inactivation via the Wanner method.

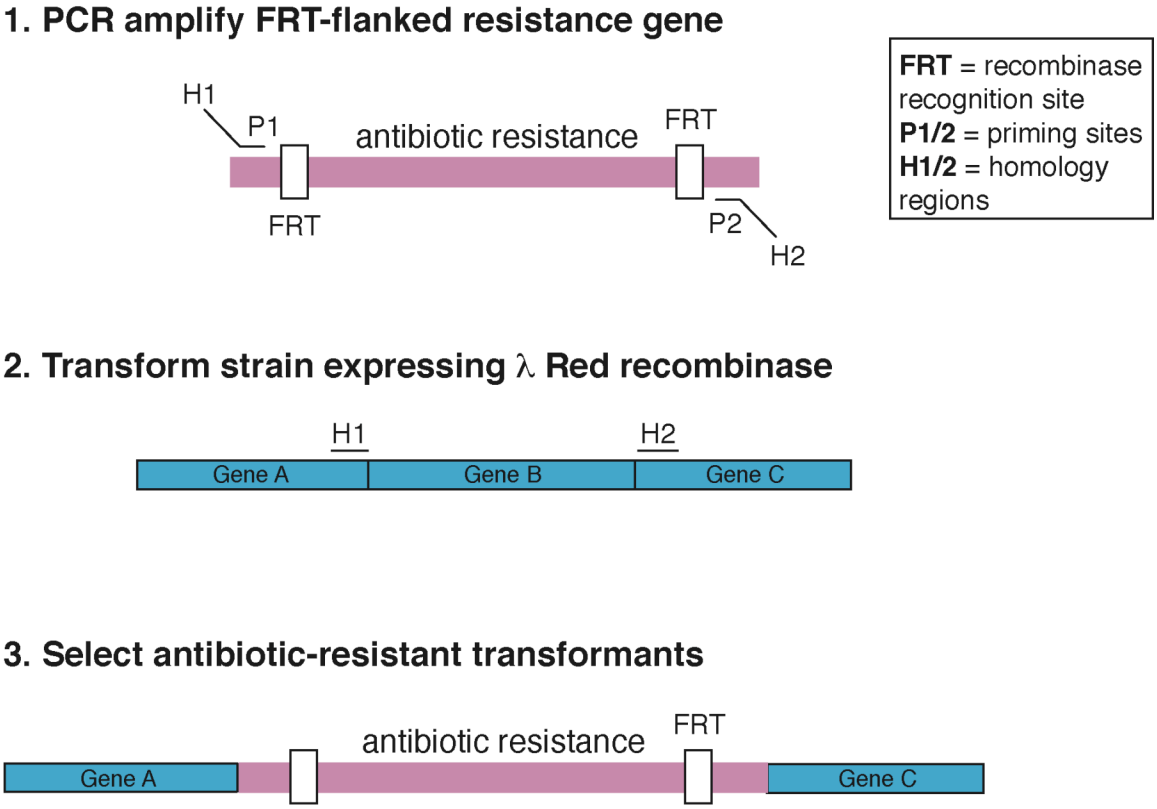


Table A2.1. Primers for Wanner inactivation of *nth* and *rnfA*.

Function	Primer Sequence
Wanner inactivation of *nth*	5'-gaagcagctgcagaacgtgcattgccaaacggtgaaacagggaatgtctg**gtgtaggctggagctgcttc**-3'
	5'-agaggataaagaaaggttatcaatggggtaatcggtgttacccctttttct**catatgaatatcctccttag**-3'
Wanner inactivation of *rnfA*	5'-ctgctctggattaacggataataggcggctttttttatttcaggccgaaaa**gtgtaggctggagctgcttc**-3'
	5'-cgccaggcccagcaggctcacggcggcaacggcaatccagatagcattca**catatgaatatcctccttag**-3'

(Stratagene). A standard 35 cycle PCR method was used with an annealing temperature of 50°C and an extension temperature of 72°C. Reaction products were loaded onto a 1.5% agarose gel and electrophoresed at 1.5 hours at 100V. Bands were excised and purified using a gel extraction kit (Qiagen). Samples were then treated with DpnI (1 μL in 50 μL total volume) for 1 hour at 37°C to remove any remaining template, purified with a QiaQuick kit (Qiagen), and concentrated.

Gene Inactivation

The target strain (CC104) was streaked to LB media and incubated overnight at 37°C. A 1 mL LB culture was started from a single colony and incubated overnight at 37°C. 200 μL of the starter culture was used to inoculate a 20 mL LB culture which was grown to OD_{600} ~ 0.4. The cells were harvested and made electrocompetent and transformed with pKD46. The transformed cells were recovered at 30°C in 1 mL LB for 3 hours. Transformants were selected on LB+amp (50 μg/mL) at 30°C. A 1 mL LB+amp (50 μg/mL) culture was grown overnight at 30°C. 200 μL of this starter culture was used to inoculate a 50 mL LB+amp (50 μg/mL) culture containing 20 mM arabinose to induce expression of the λ Red recombinase genes. Cultures were grown to OD_{600} ~ 0.4 at 30°C and harvested via centrifugation. Cells were made electrocompetent (*2*) and 15 μL of the FRT flanked resistance gene PCR product was transformed by electroporation at 2.5 kV. Cells were recovered in 1 mL LB for 3 hours at 37°C or 42°C. Recombinants were selected at on LB+kan (10 μg/mL) at 42°C. Any resulting colonies were restreaked to LB+kan and verified by colony PCR (Figure A2.2, A2.3).

Figure A2.2. PCR verification of *nth* inactivation in CC104 and CC104 *muty*-.

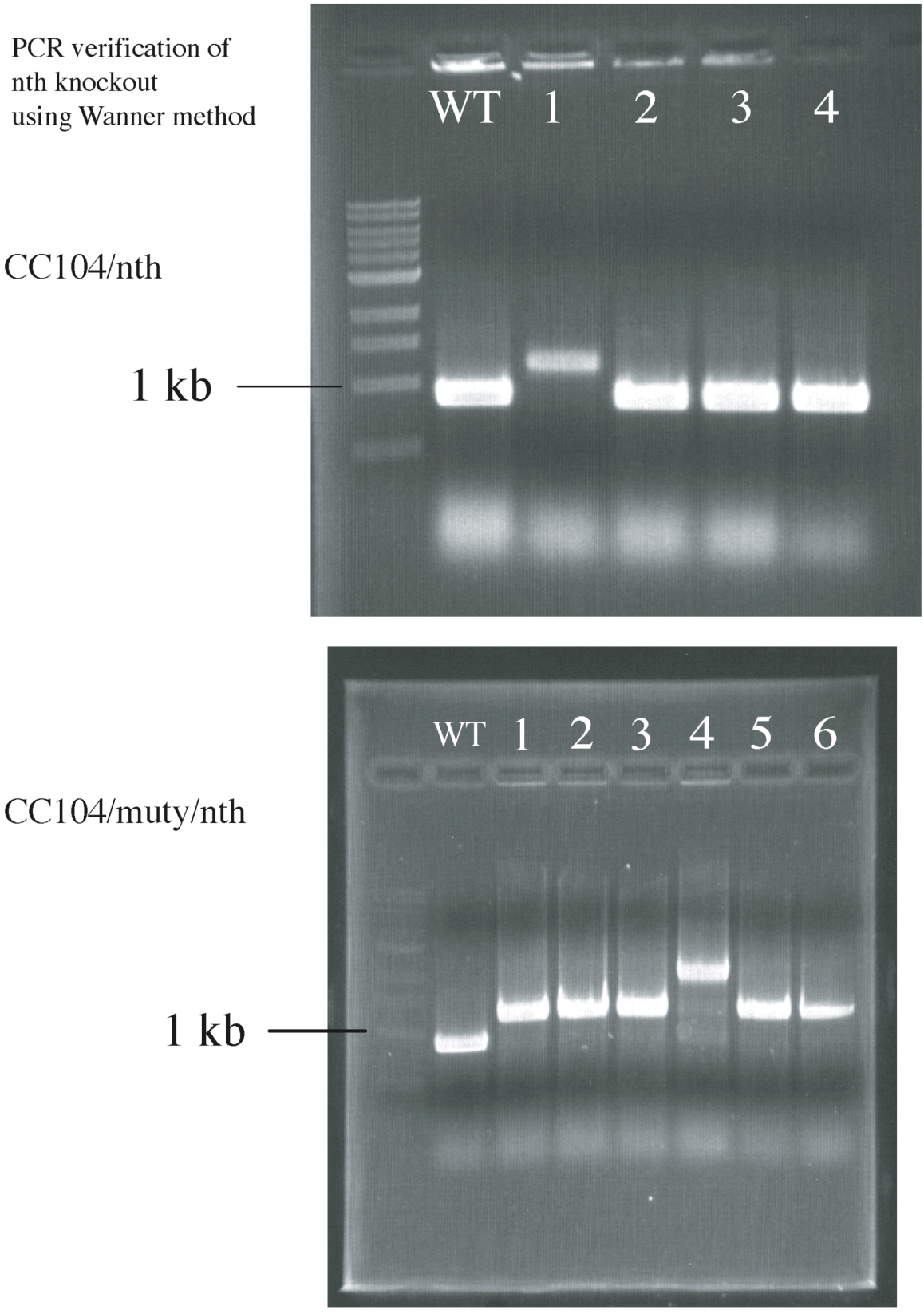
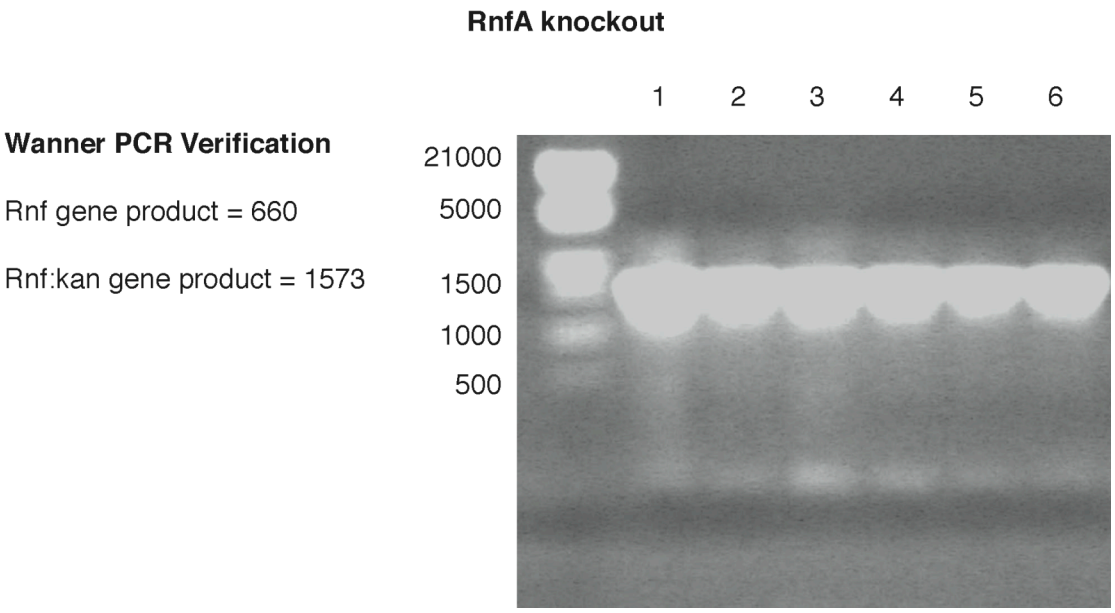


Figure A2.3. PCR verification of *rnfA* inactivation in CC102.



REFERENCES

1. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

2. Datsenko, K. A., and Wanner, B. L. (2000) *Proc. Natl. Acad. Sci. USA 97*, 6640.