Journal of Experimental Microbiology and Immunology (JEMI) Vol. 16: 90 – 95
Copyright © April 2012, M&I UBC

Construction of *Escherichia coli* K-12 Strain Deficient in *relA* and *spoT* Using the λ Red Site-Specific Recombinase System

So Ra Lee
Department of Microbiology & Immunology, University of British Columbia

Previous studies have shown that an exposure to sub-inhibitory levels of kanamycin induces capsule synthesis which confers antibiotic resistance. To examine the role of *spoT* and *relA* genes in capsule synthesis and acquired physiological antibiotic resistance during sub-inhibitory kanamycin treatment, multiple studies were performed but showed inconclusive results. A potential explanation for the lack of success in the past was the use of genetic mutants containing a number of uncharacterized mutations, which resulted in limited comparability. In this study, the λ Red recombinase system has been utilized to construct an *E. coli* K-12 Δ*relA*Δ*spoT* mutant by disrupting the *spoT* gene in *E. coli* K-12 Δ*relA* strain, JW2755-3. A PCR fragment was generated using primers with homologous sequences found upstream and downstream of the *spoT* gene, and a template plasmid, pKD3, carrying chloramphenicol resistance gene that is flanked by FLP recognition target sites. This product was electroporated into the strain JW2755-3 expressing the λ Red recombinase system. Five chloramphenicol-resistant transformants were selected and PCR-tested for the presence of new junctions and locus-specific fragments. Construct SL11W447-4 had the correct structure as evidenced by four PCR tests performed using various combinations of locus- and *cat*-specific primers. This work provides a basis for future studies.

The stringent response is a generalized stress response triggered by nutritional starvation and environmental stress (11). The stringent response causes the signaling nucleotides, also called the alarmone, ppGpp to accumulate (11). In *Escherichia coli*, the *relA* gene encodes an enzyme that carries out (p)ppGpp synthesis in response to amino acid limitation, resulting in increased synthesis of (p)ppGpp and a decrease in stable RNA synthesis (11). This response to amino acid deprivation is absent in Δ*relA* strains (9). Basal levels of (p)ppGpp synthesized in the absence of *relA* activity during balanced growth is regulated by *spoT*, which encodes an enzyme with both (p)ppGpp hydrolase and synthetase activity (11). The *spoT* gene modulates (p)ppGpp levels in response to various nutritional factors including carbon starvation (11).

Multiple studies have been conducted to investigate the roles of *relA* and *spoT* in capsule production that leads to antibiotic resistance (3, 4, 5). It has been shown that an exposure to sub-inhibitory levels of kanamycin induces capsule synthesis which confers lessened antibiotic sensitivity (3, 8). The exact roles of *relA* and *spoT* in capsule synthesis, however, still remain unclear due to inconclusive results (4, 5). One plausible explanation for the inconclusive results is that the presence of uncharacterized mutations in genetic mutants that were used in the past studies might have caused limitations in the comparability. This suggests that construction of a Δ*spoT*Δ*relA* isogenic strain bearing cleaner genetic background may improve the quality of data in future research experiments, ultimately contributing to characterization of the role of *spoT* and *relA*.

One of the best-characterized organisms in molecular biology is *E. coli* K-12 (1). In 2006, Baba *et al.* constructed the ‘Keio collection’ which is a set of precisely defined, single-gene deletions of all non-essential genes in *E. coli* K-12 (1). The important feature of these mutants is that they can be used in studies to examine any mutational effects in a common strain background (1). The ‘Keio collection’ was systematically constructed using the highly efficient gene replacement method developed in 2000 by Datsenko and Wanner (6). This method is not only useful but also straightforward to extend to other cases. The procedure is based on the λ Red recombinase system which utilizes linear PCR products that contain an antibiotic resistance cassette and homologies to the target DNA to be disrupted and its adjacent sequences (6). Being flanked by FLP recognition target (FRT) sites, the antibiotic resistance cassette can be eliminated with the FLP recombinase (6). When using this method, it is important to ensure that the deletion mutant does not demonstrate polar effects on downstream gene

Copyright © April 2012, M&I UBC

TABLE 1. *E. coli* strains and plasmids used in this study

Strains and plasmids	Characteristics	Source	Reference
JW2755-3	*E. coli* K-12 Δ*relA* strain from the KEIO Collection, *relA782(del)::kan*	Coli Genetic Stock Center at Yale University	CGSC#: 10159 (6)
MG1655/pKD46	λ Red recombinase expression plasmid, *repA101(ts), araBp-gam-bet-exo, bla(ApR)*	Laboratory Stock	CGSC#: 7669 (6)
BW25141/pKD3	Template plasmid for PCR amplification of the chloramphenicol resistance gene, *bla(ApR), FRT::cat::FRT, ori R6K* (requires the *pir* gene product for replication)	Coli Genetic Stock Center at Yale University	CGSC#: 7631 (6)

expression. Accordingly, primers should be designed so that the resulting mutant has an in-frame deletion of the target gene (1).

The objective of this study was to construct an *E. coli* K-12 Δ*relA*Δ*spoT* mutant by adapting the λ Red-based method. The *relA* deletion mutant, JW2755-3, constructed by Baba *et al.* was used as a target strain. The ultimate objective of the project was to create a Δ*relA*Δ*spoT* isogenic strain that lacked uncharacterized mutations so that it could be used in the future studies to investigate the roles of *relA* and *spoT* genes in acquired physiological antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains JW2755-3 and BW25141/pKD3, and MG1655/pKD46 as described in Table 1 were obtained from the Coli Genetic Stock Centre, Yale University and the University of British Columbia's Microbiology Laboratory stock, respectively. *E. coli* strain MG1655/pKD46 was grown in Luria-Bertani (LB) medium (1% Tryptone (Bacto #211705), 0.5% yeast extract (Bacto #212750), 1% NaCl (Fisher #S671-3)) with 100 ug/ml ampicillin (Sigma #A9518) at 30ºC. *E. coli* strain JW2755-3 was grown in LB medium with 30 ug/ml kanamycin (Sigma #K4000) at 37ºC prior to the insertion of the pKD46 plasmid. After transformation, the resulting strain JW2755-3/pKD46 was grown in LB medium with 100 ug/ml ampicillin at 30ºC. *E. coli* strain BW25141/pKD3 was used to obtain the pKD3 template plasmid and was grown in LB medium with 100 ug/ml ampicillin at 37ºC.

Plasmid isolation. Plasmids pKD46 and pKD3 were isolated from *E. coli* strains MG1655/pKD46 and BW25141/pKD3, respectively. Overnight cultures were performed in 3 ml LB medium supplemented with appropriate antibiotics and incubated with mild aeration at 30ºC. Plasmids were then isolated using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen #K2100) according to the manufacturer's protocol.

PCR primers. Primers used for amplifying the chloramphenicol resistance (*cat*) gene were based on the sequence provided by Baba *et al* (1). Forward primers (H1P1-f-SL-MICB 447-11w; hereafter abbreviated as H1P1) consisted of 50-nt 5' ends homologous to upstream *spoT* sequence (H1) and the 20-nt 3' ends for priming upstream (P1) of the FRT sites flanking the *cat* gene in pKD3 (see Table 2 for primer sequences). Reverse primers (H2P2-r-SL-MICB 447-11w; hereafter abbreviated as H2P2) had 50-nt 5'ends homologous to downstream *spoT* sequence (H2) and the 20-nt 3' ends for priming downstream (P2) of the FRT-flanked *cat* gene. In order to confirm upstream and downstream junctions in recombinants as well as to verify simultaneous loss of the *spoT* gene and gain of the *cat* gene, four PCR primers were used: the common test primers for *cat* (c1- r-SL-MICB 447-11w and c2-f-SL-MICB 447-11w; hereafter abbreviated as c1 and c2, respectively) and nearby locus-specific primers (U-f-SL-MICB 447-11w and D-r-SL-MICB 447-11w; abbreviated as U and D, respectively) which were designed using the Primer3: WWW Primer Tool (University of Massachusetts Medical School, U.S.A.).

Generation of PCR fragments. PCR reactions were carried out in 50 ul reactions containing 2.5 U of *Taq* DNA polymerase (Invitrogen #18038-042), 1 X PCR buffer, 1 ng pKD3 DNA, 1.0 uM of each primer (H1P1 and H2P2), 200 uM dNTPs, and 1.5 mM $MgCl_2$. The gradient PCR machinie was used to carry out the reaction. The PCR program was as follows: 95°C for 5 min, followed by 35 cycles at (i) 94°C for 30 s, (ii) 65°C for 30 s, (iii) 72°C for 2 min, plus an additional 5 min at 72ºC. PCR products were digested with *Dpn*I in NEBuffer 4 (to eliminate methylated/unamplified template DNA), followed by ethanol precipitation and resuspension in 6 ul dH_2O.

Preparation of electrocompetent cells. This method was adapted from previous studies (2). For preparation of electrocompetent *E. coli* JW2755-3, 5 ml LB medium supplemented with 30 ug/ml kanamycin was inoculated with a well-isolated colony and incubated overnight at 37ºC. For preparation of electrocompetent *E. coli* JW2755-3/pKD46, 5 ml LB supplemented with 100 ug/ml ampicillin was inoculated with a well-isolated colony and incubated overnight at 30ºC. On the next day, 1 ml of the overnight culture was added to 100 ml (1:100 dilution) SOB medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, and 20 mM $MgSO_4$) supplemented with 30 ug/ml kanamycin (for JW2755-3) or SOB supplemented with 100 ug/ml ampicillin and 10 mM L-(+)-arabinose (Sigma #A3256) induction (for JW2755-3/pKD46). The cultures were incubated in the 30ºC shaker until an OD_{600} of 0.35-0.40 and immediately chilled on ice for 20 min, followed by centrifugation at 1000*g* for 20 min at 4ºC. The supernatant was discarded and the resulting cell pellets were washed twice in 50 ml ice cold ddH_2O, then once in 10 ml 10% glycerol, and another time in 5 ml ice cold 10% glycerol. The pellets were then resuspended in 0.5 ml of ice cold 10% glycerol.

Electroporation and mutant selection. This method was adapted from previous studies (2). A measure of 50 ul of electrocompetent cells (JW2755-3/pKD46) was mixed with 400 ng of the PCR fragment in a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad #165-2086). Cells were electroporated at 2.5 kV using a Bio-Rad GenePulser (Bio-Rad #165-2105), immediately followed by the addition of 1 ml of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, and 20 mM glucose) with 1 mM L-arabinose. The suspension was then transferred to a metal capped 16 x 125 mm test tube and incubated at 30°C for 2 hours. After recovery, 0.1 ml of the cell suspension was spread onto an agar plate containing 25 ug/ml chloramphenicol (Sigma #C0378).

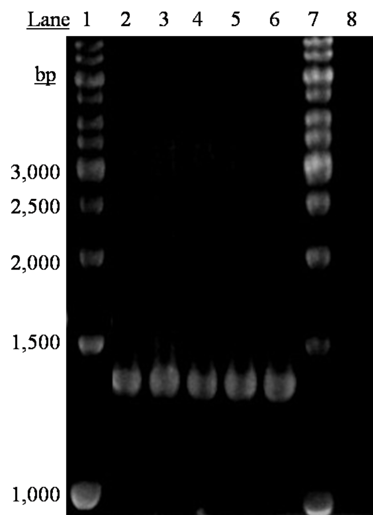

FIG. 1. The PCR products generated using locus-specific primer U and H2P2 to verify the deletion of *spoT* gene in five selected mutants (SL11W447-1~5). Lanes 1 and 7 are the 1 kb molecular weight ladders. Lanes 2-6 are the PCR fragments produced using U and H2P2 primers on the five selected mutants SL11W447-1 – 5 (1.3 kb) while Lane 8 is the negative control (JW2755-3/pKD46).

PCR verification of deletions. This method has been adapted from Datsenko and Wanner (6). Various PCR reactions were carried out to test that the mutants had the correct chromosomal structures. Five independent colonies were selected and suspended in 1 ml LB medium with chloramphenicol and incubated overnight at 30°C. A volume of 1 ul of each culture was separately examined in 20-ul PCR reactions. To test for both new junction fragments, two reactions were performed using primers c1 with U (expected fragment size: 468 bp) and c2 with D (430 bp). To verify simultaneous loss of the *spoT* gene and gain of the FRT-flanking *cat* gene, a third reaction was carried out using primers U and D (1,607 bp) or U and H2P2 (1,337 bp). PCR products were analyzed by 1.5% agarose gel electrophoresis (at 100 V for 1 hr) using 1 X TBE and GeneRuler™ 1kb DNA ladder (Fermentas #00033708) or Low DNA Mass Ladder (Invitrogen #10068-013).

RESULTS

PCR tests were performed on several representative mutants using locus-specific and/or *cat*-specific primers to confirm the presence of new junctions and locus-specific fragments of the expected sizes. Figure 1 shows the results of the agarose gel analysis performed on various PCR fragments generated independently using U and H2P2 primers. Five selected transformants, labeled SL11W447-1 through 5, were shown to give the expected band size of 1.3 kb when PCR amplification was performed using the primers U and H2P2 (lanes 2-6). The strain JW2755-3/pKD46, which was used as the negative control, showed no bands as expected since the presence of *spoT* gene in that strain prevented the PCR reaction when the same set of primers were used (lane 8). Therefore, my data suggests that all five of the chloramphenicol-resistant transformants tested had the *cat* gene integrated into the correct location on the chromosome.

Figure 2 shows the result of another PCR test performed using locus-specific primers, U and D, on the five mutants to determine whether their *spoT* genes had been successfully disrupted. The results confirmed the gene disruption; the expected product (1.6 kb) was observed for the five mutants (lanes 2-4) and a heavier band (lane 7) for the strain JW2755-3/pKD46. The latter strain gave a heavier product due to the presence of *spoT* gene, which is bigger than the *cat* gene. However, these results seen in Fig. 2 were not as clean as the one shown in Fig. 1. Along with the expected

TABLE 2. Primers used in PCR

Primer name	Description	Sequence in 5′ to 3′ direction (length)	Reference
H1P1-f-SL-MICB 447-11w	Forward primer used for Cm^R cassette amplification	TTACCGCTATTGCTGAAGGTCGTC GTTAATCACAAAGCGGGTCGCCCT TGgtgtaggctggagctgcttc† (70 nt)	(1)
H2P2-r-SL-MICB 447-11w	Reverse primer used for Cm^R cassette amplification	CGTGCATAACGTGTTGGGTTCATA AAACATTAATTTCGGTTTCGGGTG ACatgggaattagccatggtcc† (70 nt)	(1)
c1- r-SL-MICB 447-11w	*cat*-specific primer used for verification of upstream junction	TTATACGCAAGGCGACAAGG (20 nt)	(6)
c2-f-SL-MICB 447-11w	*cat*-specific primer used for verification of downstream junction	GATCTTCCGTCACAGGTAGG (20 nt)	(6)
U-f-SL-MICB 447-11w	*rpoZ*-specific primer used for verification of upstream junction	CCGTTTTGACCTGGTACTGG (20 nt)	
D-r-SL-MICB 447-11w	*trmH*-specific primer used for verification of downstream junction	AGATGGGTTGCCAGAATCTG (20 nt)	

† Capital letters correspond to external overlap with the *spoT* gene while lowercase letters indicate internal overlap with the chloramphenicol resistance marker.

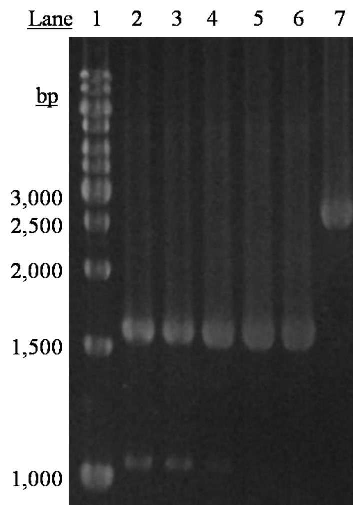

FIG. 2. The PCR products generated using locus-specific primers U and D to verify the deletion of *spoT* gene in five selected mutants (SL11W447-1~5). Lane 1 is the 1 kb molecular weight ladder. Lanes 2-6 are the PCR fragments produced using U and D primers on the mutants SL11W447-1 – 5 (1.6 kb) while Lane 7 is the one generated using the same primers on JW2755-3/pKD46 (2.7 kb). An extra faint band (~1.1 kb) is also observed in lanes 2-4.

products (1.6 kb), an extra faint band (~1.1 kb) was observed in three of the tested mutants - SL11W447-1 – 3 (lanes 2-4).

The five transformants were further tested for new junction fragments by using a combination of locus-specific (U and D) and *cat*-specific primers (c1 and c2) as shown in Figure 3. The mutants SL11W447-1 (lanes 2 and 9) and SL11W447-2 (lanes 3 and 10) gave unexpected results such as showing no bands or an extra band (~1.1 kb), unlike the other three mutants which gave the expected products of upstream (468 bp) and downstream junctions (430 bp). The negative controls showed no bands (lanes 7 and 14). Assuming that the unclear results observed for the two mutants were due to the use of relatively older culture stocks, which might have contained degraded or sheared DNA, the colony PCR reactions were re-performed using fresher culture. The results for the repeated test are shown in Figure 4. Yet again, the extra band of the same size as previously observed (~1.1 kb) was seen in the first three mutants, SL11W447-1 – 3 (lanes 2-4). Likewise, the mutants SL11W447-1 and SL11W447-5 did not show the bands corresponding to the downstream junction (lanes 9 and 13).

DISCUSSION

In order to check for the mutants with the correct structure and successful knockout of the *spoT* gene, four PCR tests were performed using different combinations of the primers. Various data were obtained from the tests but taken all together, at least one of the five tested transformants, SL11W447-4, appeared to show the expected positive results in all four PCR tests performed. Hence, my data verifies that the mutant SL11W447-4 has the predicted structure.

As mentioned in the Results section, non-specific bands were seen in some PCR samples. One interesting observation to note is that the non-specific products tend to show a gradient in their concentrations; the first band seems to be the brightest and it gets lighter across the lane (lanes 2-4 in Fig. 2 and lanes 2-4 in Fig. 4). This trend seems to have arisen from the procedure in which the PCR mix was added to the colony suspension. Due to the lack of practice and caution, I was not aware that the PCR reaction needs to be assembled on ice. Because most of the reagents for PCR mix were left at room temperature when being added, it is highly probable that this had caused unwanted reactions to occur during the preparation step. A few single-stranded DNAs present in the colony mixture might have reacted with the polymerase prior to the start of the reaction. Although this non-specific reaction must have been present in low amounts initially, its quantity would have been sufficient to cause amplification

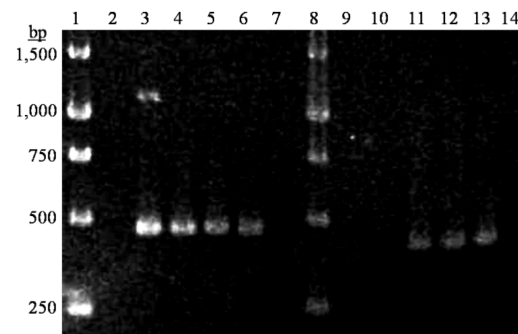

FIG. 3. The PCR products generated using primers U and c1 or D and c2 to confirm the presence of two new junctions in five selected mutants (SL11W447-1~5). Lanes 1 and 8 are the 1 kb molecular weight ladders. Lanes 2-6 are the results of PCR amplification using U and c1 primers on the five selected mutants SL11W447-1 – 5 while Lane 7 is the negative control (JW2755-3/pKD46). Similarly, lanes 9-13 are the results of PCR amplification using D and c2 primers on the five mutants while Lane 14 is the negative control.

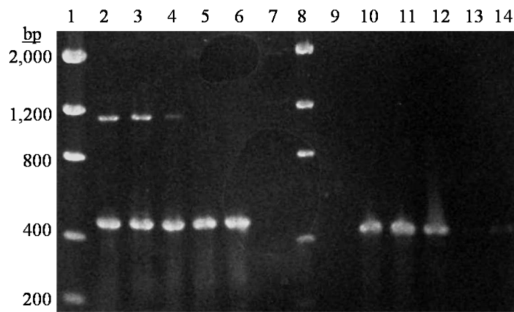

FIG. 4. The repeat of the PCR test using primers U and c1 or D and c2 on five selected mutants (SL11W447-1~5). Lanes 1 and 8 are the low DNA mass ladder. Lanes 2-6 are the results of PCR amplification using U and c1 primers on the five selected mutants SL11W447-1 – 5 while Lane 7 is the negative control (JW2755-3/pKD46). Similarly, lanes 9-13 are the results of PCR amplification using D and c2 primers on the five mutants while Lane 14 is the negative control.

during the cycling period. This may explain the gradient appearing in the non-specific bands, which can correlate with the delay time caused between samples during the preparation step. The occurrence of non-specific bands could be minimized if future experiments ensure that PCR mix preparation steps are correctly performed. Moreover, using hot start PCR method would help to eliminate formation of non-specific products prior to high-temperature cycling. The hot start method can be performed by heating the reaction components to the denaturation temperature before adding the polymerase or by using specialized enzyme systems that inhibit the polymerase's activity at ambient temperature but activate it after a high-temperature step (7).

The ultimate goal of this project is to allow future researchers to use the newly constructed double knockout strain lacking in both *relA* and *spoT* genes for studying the role of two genes. The targeting PCR products in this study were designed to create an in-frame deletion, leaving the start and stop codons of *spoT* gene and translational signal for a neighbouring downstream gene, *trmH* gene, intact upon excision of the resistance cassette. However, the *spoT* disruption mutant could be containing 1-nt deletion as noted by Datsenko and Wanner (6). In their study, thirteen different gene disruption mutants were verified by a PCR test. As further verification, they sequenced the locus-specific fragments and reported that around 10% of the tested had 1-nt deletions (6). They suggested that the incorrect ones resulted from PCR products generated from a primer with 1-nt deletions, which occurred at or very near the junction of a priming site and homology extension. If a single nucleotide deletion were made within the *spoT* knockout construct, this would interfere with the in-frame deletion and lead to a frameshift mutation in the downstream *trmH* gene, which is essential for tRNA (Gm18) methyltransferase activity (10). However, previous studies have shown that the absence of Gm18 in tRNA has no significant effect on bacterial growth or on the efficiency of decoding during translation (10, 12). Hence, the deletion of one nucleotide in the construct would likely cause no major disturbance of cell function.

In conclusion, I have isolated the *spoT* gene disruption mutant by direct transformation of *E. coli* carrying a Red helper plasmid with PCR products having homology extensions for the targeted locus. Based on the gel electrophoresis data, the mutant SL11W447-4 yielded the predicted fragment sizes in all four PCR tests performed in this study, indicating that the mutant SL11W447-4 has the correct chromosomal structure. In spite of the inconsistencies seen in the results, the construction of the *E. coli* K-12 Δ*relA*Δ*spoT* mutant was successfully achieved.

FUTURE EXPERIMENTS

Continuation of this work should examine the initial question about the effect of *relA* and *spoT* on the capsule production that confers antibiotic resistance. This work has successfully demonstrated that the construct SL11W447-4 can be used as a representative double knockout strain in future experiments. If required, the FRT-flanked antibiotic resistance genes, *kan* and *cat*, can be eliminated with the FLP recombinase (6). In order to do so, the *spoT* mutants should be transformed with plasmid pCP20 which is a helper plasmid encoding the FLP recombinase (6). After selecting for ampicillin-resistant clones, additional plating can be performed to assay for loss of all antibiotic resistance. This will result in a clean *spoT* knockout strain.

ACKNOWLEDGMENTS

This study was funded by the Department of Microbiology and Immunology at the University of British Columbia. I would like to thank Dr. William Ramey for his patience, guidance, and support throughout the course of the project. I would also like to thank Lando and Nick for providing clean glassware.

REFERENCES

1. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, and H. Mori**. 2006. Construction of *Escherichia coli* K-12 in-frame, single-

Copyright © April 2012, M&I UBC

gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2**:1-11.

2. **Beamish, L., R. Greenwood, K. Petty, and E. Preston**. 2004. Successful application of the λ-red recombinase system to inactivate *lacI* in *Escherichia coli* C29, BW25993 and MG1655. J. Exp. Microbiol. Immunol. **12**:94-99
3. **Cheng, K., L. Ren, and K. Tsai**. 2009. Inhibitory concentrations of kanamycin in the presence of ppGpp synthase RelA confer protection against subsequent lethal antibiotic assaults in *E. coli* CP78. J. Exp. Microbiol. Immunol. **14**:51-56.
4. **Chenne, W., L. Ng, and M. Pambid**. 2010. Assessing the role of SpoT and RelA in capsular polysaccharide synthesis after treatment with sub-lethal concentrations of kanamycin to confer decreased antibiotic sensitivity in *Escherichia coli*. J. Exp. Microbiol. Immunol. **15**:52-58.
5. **Chiang, V., B. Wong, C. Wong, and J. Yu**. 2010. On the limited role of *relA* in kanamycin and amino acid starvation induced stringency and subsequent antibiotic cross-protection in *Escherichia coli.* J. Exp. Microbiol. Immunol. **15**:59-63.
6. **Datsenko, K.A., and B.L. Wanner**. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. **97**:6640–6645.
7. **Lebedev, A.V., N. Paul, J. Yee, V.A. Timoshchuk, J. Shum, K. Miyagi, J. Kellum, R.I. Hogrefe, and G. Zon.** 2008. Hot start PCR with heat-activatable primers: a novel approach for improved PCR performance. Nucleic Acids Res. **36**:e131.
8. **Liu, H., M. Zhu, and S. Zhu**. 2010. Persistence of antibiotic resistence and capsule in *E. coli* B23 after removal from sublethal kanamycin treatment. J. Exp. Microbiol. Immunol. **15**:43-46.
9. **Metzger, S., I. B. Dror, E. Aizenman, G. Schreiber, M. Toone, J. D. Friesen, M. Cashel, and G. Glaser.** 1988. The nucleotide sequence and characterization of the *relA* gene of *Escherichia coli*. J Biol. Chem. **263**:15699–15704.
10. **Persson, B.C., G. Jäger, and C. Gustafsson.** 1997. The *spoU* gene of *Escherichia coli*, the fourth gene of the *spoT* operon, is essential for tRNA (Gm18) 2'-O-methyltransferase activity. Nucleic Acids Res. **25**:4093–4097.
11. **Potrykus, K., and M. Cashel**. 2008. (p)ppGpp: still magical?. Annu. Rev. Microbiol. **62**:35-51.
12. **Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem. **266**:5980-90.