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An improved counterselectable marker system for mycobacterial recombination using *galK* and 2-Deoxy-Galactose

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Abstract

Counterselectable markers are powerful tools in genetics because they allow selection for loss of a genetic marker rather than its presence. In mycobacteria, a widely used counterselectable marker is the gene encoding levan sucrase (*sacB*), which confers sensitivity to sucrose, but frequent spontaneous inactivation complicates its use. Here we show that the *E. coli* galactokinase gene (*galK*) can be used as a counterselectable marker in both *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Expression of *E. coli galK*, but not the putative *M. tuberculosis galK*, conferred sensitivity to 2-deoxy-galactose (2-DOG) in both *M. smegmatis* and *M. tuberculosis*. We tested the utility of *E. coli galK* as a counterselectable marker in mycobacterial recombination, both alone and in combination with *sacB*. We found that 0.5% 2-DOG effectively selected recombinants that had lost the *galK* marker with the ratio of *galK* loss/*galK* mutational inactivation of approximately 1:4. When we combined *galK* and *sacB* as dual counterselectable markers and selected for dual marker loss on 0.2% 2-DOG/5% sucrose, 98.6–100% of sucrose/2-DOG resistant clones had undergone recombination, indicating that the frequency of mutational inactivation of both markers was lower than the recombination frequency. These results establish a new counterselectable marker system for use in mycobacteria that can shorten the time to generate unmarked mutations in *M. smegmatis* and *M. tuberculosis*.

Keywords

Galactokinase; counterselectable markers; Recombination; Tuberculosis

1. Introduction

Counter-selectable markers, i.e. genes whose presence can be selected against, are a powerful tool in molecular microbiology and cell biology. Uses of counterselectable markers in genetics include selection of recombination products, selection for plasmid loss, and as reporter genes (Reyrat et al., 1998). Despite advances in recent years in techniques for genetic manipulation of mycobacteria, several deficiencies still remain. Although several

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reliable methods are available to disrupt chromosomal genes with markers conferring antimicrobial resistance, allelic exchange of unmarked deletion mutations or amino acid substitution alleles is still laborious. One frequently used method for construction of unmarked mutations utilizes a two step strategy in which resolution of a tandem chromosomal duplication (produced through recombination of a suicide plasmid at the locus of interest) is selected through loss of a counterselectable marker. Two markers have been widely used for this purpose: *rpsL* and *sacB*. *rpsL* has been useful, but requires a background strain that is streptomycin resistant, limiting its general utility (Sander et al., 1995). *sacB*, which confers susceptibility to sucrose (Pelicic et al., 1996a), has proven extremely useful in the genetic manipulations mentioned above (Pelicic et al., 1996b; Pavelka and Jacobs, 1999). However, *sacB* has a frequency of spontaneous inactivating mutations that is higher than that of the desired targeted recombination event. In published use of *sacB* in *M. smegmatis* recombination, 60–70% of sucrose resistant cells are due to *sacB* inactivation rather than recombination (Pavelka and Jacobs, 1999). Additional counter-selectable markers are therefore needed, either with a lower spontaneous inactivation rate, or for combined use with *sacB*.

The *Escherichia coli* (*E. coli*) *galK* gene (*galK*), encoding the enzyme galactokinase, catalyzes the phosphorylation of galactose to galactose-1-phosphate. It also efficiently phosphorylates a galactose analogue, 2-deoxy-galactose (2-DOG). The product of this reaction, 2-deoxy-galactose-1-phosphate, cannot be further metabolized, leading to buildup of toxic levels and cell death. Thus, the constitutive expression of *galK* in *E. coli* leads to sensitivity to 2-DOG, but is otherwise nontoxic to the cell, a property that has been used for counterselection in *E. coli* (Alper and Ames, 1975; Ueki et al., 1996; Warming et al., 2005). The small size of the *galK* gene is also advantageous in PCR amplification and other genetic manipulations. We therefore attempted to adapt *galK* for use as a counter selectable marker in mycobacteria, specifically *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*.

2. Materials and methods

2.1 Strains and media

M. smegmatis mc²155 was grown in liquid 7H9 media, supplemented with 0.05% Tween 80, 0.5% glycerol and 0.5% dextrose. For solid media, 7H10 was used with same additives but without Tween. *M. tuberculosis* (Erdman) was grown on 7H10 plates with 10% OADC, 0.5% glycerol. When needed, 2-deoxy-galactose (2-DOG) (Sigma Aldrich, D4407) was added at 0.2 or 0.5%. 5% Sucrose was added when needed. Antibiotics used were kanamycin 40 g/ml for *E. coli* or 20 g/ml for mycobacteria, and hygromycin B at 150 g/ml for *E. coli* and 100 g/ml for mycobacteria. Plasmids were introduced into mycobacteria by electroporation (Bio-Rad gene pulser II, 25 µF, 1000 Ohms, 2.5V). All growth was at 37°C.

2.2 *galK* cloning

To constitutively express mycobacterial *galK* (Rv0620, *mycgalK*) from the MOP (mycobacterial optimized promoter) promoter, we PCR amplified it from *Mycobacterium tuberculosis* (Strain Erdman) genomic DNA using primers oDB71 (5'–**CAGATCTGCCCATGACGGTCAGCTAC**–3' and oDB72 (5'–**ACTGCAGACCCGCTCAACGGCACTC**–3'). The product was cut with BglII and PstI (enzyme sites in bold in primer sequence), and cloned into the same sites in a previously constructed plasmid named pDB41 (a plasmid with kanamycin and ampicillin resistance markers, no mycobacterial origin of replication, and a Mycobacterial Optimized Promoter (MOP)) placing it under the control of that strong promoter. The resulting plasmid, pDB56, was cut with EcoRI and PstI, and the 752 bp PstI/EcoRI piece from pMV306kan, containing the *attP* site, was inserted to create pDB58.

To express *E. coli galK* in mycobacteria, we PCR amplified it from chromosomal DNA of *E. coli DH5-alpha* using primers oDB77 (5'-CAGATCTTCCGGAGTGTAAGAAATGAGTC-3') and oDB78 (5'-ACTGCAGGAGTTTCGTTTCAGCACTGTC-3'). The correct sequence of the product was confirmed, and it was then cloned into the Bgl II and PstI sites in the previously described pDB58, replacing the *mycgalK*. The resulting vector is called pDB77 (Fig 1, left). Both pDB58 and pDB77 have an *attP* site, by which they can be integrated as a single copy into the chromosome of mycobacteria by the action of the L5 integrase (Lee et al., 1991), acting either in *cis* or *trans*. pDB58 and pDB77 have no integrase gene, and therefore were integrated by in *trans* expression of integrase (from a previously integrated plasmid, replaced by pDB58 or pDB77) to avoid subsequent plasmid excision.

2.3 Allelic exchange at the *ligD* and *rpoB* loci

For deletion of *ligD*, we used pMSG346, a plasmid previously used to delete *ligD* from *M. smegmatis* in a 2 step allelic exchange (Aniukwu et al., 2008), and contains the 5' and 3' flanking regions of *ligD* adjacent to each other. We cloned *galK* from pDB77 (NsiI, PstI) into pMSG346 (SbfI), creating pDB88 (Fig 1, right). The 5' flanking region of *ligD* was used as a probe for Southern blotting to confirm allelic exchange at the *ligD* locus. For the deletion of *rpoB*, the 5' and the 3' flanking regions of *rpoB* were cloned into pDB88 instead of the flanking regions of *ligD*. The 5' region was PCR amplified from *M. smegmatis* genomic DNA using primers: (5'-GCTCGAGCCGCGATTTCGGCGATTTTCTCC-3') and (5'-GTCTAGACACCTCATGCGACTATTCGGATGC-3'). The 3' prime flank was PCR amplified using primers: (5'-GTCTAGAGAGCCTCGTCCGAAGACCG-3') and (5'-GTCTAGACGAGCTTGGTGAAGGTGTTCCAG-3'). The resulting plasmid was named pCLS1.

3. Results

3.1 Expression of *E. coli*, but not *M. tuberculosis*, *galK* confers sensitivity to 0.2% 2-DOG in *M. smegmatis* and *M. tuberculosis*

Although mycobacteria have a native *galK* gene (*Rv0620*, *MSmeg_3692*), it is under tight regulation and requires glutamate for induction (Raychaudhuri et al., 1998). We postulated that constitutive expression of mycobacterial *galK* might render the bacteria 2-DOG sensitive. To test this idea, we expressed *Rv0620* from the constitutive MOP promoter from the *attB* site on the *M. smegmatis* chromosome. Importantly, pDB58 has an *attP* site, by which it can be integrated as a single copy into the chromosome of mycobacteria, but has no integrase gene, and therefore it can only be integrated into bacteria that expresses the integrase gene by means of marker exchange (Pashley and Parish, 2003). However, once integrated, the cassette cannot spontaneously be excised, because of that very same lack of integrase. This integration strategy should minimize loss of the plasmid encoding *galK* as a reason for growth on 2-DOG. The resulting strain was named MGM1959. We tested MGM1959 for its ability to grow on 7H10 plates supplemented with 0.2% 2-DOG by plating serial ten-fold dilutions. We found 2-DOG did not inhibit the growth of MGM1959, either in agar media (Figure 2) nor in liquid media (not shown). Concentrations of 2-DOG of up to 2% were used with no apparent inhibition of growth (data not shown). Thus, it does not appear that expression of mycobacterial *galK* confers sensitivity to 2-DOG. Several explanations are possible for this result including lack of uptake of 2-DOG into mycobacteria, lack of toxicity of 2-DOG-1-phosphate in mycobacteria even if produced, or inefficient use of 2-DOG by mycobacterial galactokinase.

To examine these possibilities, we next tested whether heterologous expression of *E. coli galK* could confer sensitivity to 2-DOG in mycobacteria. We constructed an *M. smegmatis*

strain expressing *E. coli galK* (*ECgalK*) from the MOP promoter, using pDB77 (Figure 1). This strain was named MGM1970. We tested MGM1970 and a control strain carrying an *attB* integrated vector conferring hygromycin resistance (*M. smegmatis* + pYUB412) for their ability to grow on 7H10 agar media with or without 0.2% 2-DOG. We found 2-DOG did not interfere with growth of wild type *M. smegmatis*, but almost completely inhibited the growth of MGM1970 (Fig 3A). To better quantify the ability of 0.2% 2-DOG to inhibit growth of bacteria expressing *E. coli galK*, we plated 10-fold dilutions of MGM1970 on 7H10 plates with or without 0.2% 2-DOG. We first observed a single 2-DOG resistant colony when plating 10^5 bacteria (Figure 3B), with heavier growth when 10^6 – 10^7 bacteria were plated. Thus, the efficiency of selection is approximately 1 in 10^5 . These results indicate that 2-DOG is toxic to mycobacteria when *E. coli galK* is expressed and suggest that our failure to observe 2-DOG sensitivity with mycobacterial *galK* is likely due to inefficient use of 2-DOG by the enzyme.

We also tested different concentrations of 2-DOG to optimize counterselection. We plated approximately 1000 *M. smegmatis attB::ECgalK* (MGM1970) on 7H10 plates with 0, 0.05%, 0.1% or 0.2% 2-DOG, and observed growth after 72 hours. Almost no selection was observed at the 0.05% concentration, but at 0.1% there was substantial inhibition of growth – although the number of colonies was not decreased, their growth rate was much reduced. At 0.2% there was, as expected, no growth (Fig 4A).

To test whether the 2-DOG/*galK* system is also effective for counter selection in slow growing pathogenic mycobacteria, we constructed an *M. tuberculosis* strain carrying pDB77 (MGM1971), and compared its growth to wild type *M. tuberculosis* on agar media containing 0.2% 2-DOG. As was found in *M. smegmatis*, we found no effect of 0.2% 2-DOG on WT bacteria, but almost complete inhibition of MGM1971 (Fig 4B). We therefore conclude that expression of *E. coli galK* confers sensitivity to 0.2% 2-DOG in *M. tuberculosis* as well.

3.2 Use of *galK*/2-DOG as a counterselectable marker in mycobacterial recombination

The experiments above demonstrate that *E. coli galK* confers sensitivity to 2-DOG in mycobacteria, suggesting that this marker may be useful to enrich for recombinants in genetic strategies using two step allelic exchange. *ligD* is a nonessential gene that was previously deleted from *M. smegmatis* using a 2 step allelic exchange strategy with the *sacB* marker (Gong et al., 2005) and therefore served as a useful genetic locus to test the *galK* system. To test *sacB* and *galK* simultaneously, we constructed pDB88, a plasmid with the 3' and 5' flanking region (each 500bp long) of *ligD*, *sacB*, *E. coli galK* and a hygromycin resistance cassette (Fig 1). Note that this plasmid does not contain a mycobacterial origin of replication and therefore, in mycobacteria, hygromycin resistance can only be conferred through integration of the plasmid into the chromosome through homologous recombination of one of the flanking regions of *ligD* with the native *ligD* locus, or illegitimate recombination elsewhere in the chromosome. A single targeted crossover integration event produces a chromosomal duplication in which the native *ligD* locus is separated from the *ligD* deletion allele by intervening plasmid sequences, which in this case contain the hygromycin resistance marker, *sacB*, and *galK*. As such, this strain is hygromycin resistant and sucrose/2-DOG sensitive. A map of the chromosomal duplication arising from recombination at the 3' flanking region of *ligD* is shown in Figure 5A, along with possible resolution events to regenerate the wild type *ligD* allele (Figure 5B) or produce a *ligD* deletion (Figure 5C). The resolution products are sucrose/2-DOG resistant and hygromycin sensitive. Mutational inactivation of *galK*, *sacB*, or both could also produce resistance to 2-DOG or sucrose, but these events are discerned by their hygromycin resistance and by Southern hybridization (Fig 5D).

We electroporated pDB88 into *M. smegmatis*, and selected a single hygromycin resistant transformant which had undergone recombination via the 3' flanking region of *ligD* (Fig 6A,B–left lanes). Removal of hygromycin selection allowed a second targeted recombination event to occur. This second recombination event between one of the *ligD* flanking regions will delete the intervening plasmid sequences, resulting in a hygromycin sensitive, sucrose and 2-DOG resistant cell (Fig 5). To test the relative utility of sucrose, 2-DOG, or sucrose/2-DOG counterselection, we plated serial dilutions of the bacteria on plates containing 5% sucrose, 0.5% 2-DOG, or 5% sucrose and 0.2% 2-DOG. We characterized the colonies arising from these counterselections by testing them for hygromycin resistance and further confirming recombination by Southern blotting. By combining the results of two separate experiments (Table 1, top panels), we tested 96 colonies surviving counterselection on 0.5% 2-DOG, 135 colonies surviving counterselection on 5% sucrose, and 56 colonies surviving counterselection on 0.2% 2-DOG/5% sucrose. Of the bacteria counterselected on 0.5% 2-DOG, 16 out of 96 colonies (16.7%) were hygromycin sensitive, confirming a precise second recombination event. This was in contrast to the group counterselected on 5% sucrose, in which only 3 out of 135 colonies (2.2%) were hygromycin sensitive. Out of 56 colonies that survived counterselection on 2-DOG and sucrose, all were sensitive to hygromycin, suggesting all had undergone a precise second-recombination event (Table 1, upper panels).

To confirm that the sucrose/2-DOG resistant, hygromycin sensitive, colonies had undergone a precise second recombination event, we probed genomic DNA by Southern blotting. All 7 colonies identified in the 0.5% 2-DOG screen were examined, as were 9 of the 39 identified by combination screening on 0.2% 2-DOG/5% sucrose. The Southern blot confirmed all colonies were indeed true precise recombination events, with even distribution between deletion mutants and reversal to WT (Fig 6B).

To further substantiate our results, we attempted to delete the essential *rpoB* gene from *M. smegmatis*. For this experiment, the bacteria were electroporated with pCLS1, a pDB88-like plasmid with the flanking regions of *rpoB* inserted in the same location as the *ligD* deletion allele. The bacteria were selected on hygromycin, a resulting colony was grown, diluted into fresh media with no antibiotic, allowed to grow overnight, and plated on either 0.2% 2-DOG/5% sucrose or 5% sucrose alone. 75 out of 76 colonies (98.7%) from the double counter selection group were hygromycin sensitive, suggesting a precise recombination event, whereas only 7 out of 130 (5.38%) sucrose resistant clones were hygromycin sensitive, (table 1, lower panels). 7 randomly chosen colonies from the double counter selection group were also analyzed by Southern blotting. Although none of these were found to be a deletion mutant, they were all confirmed as a precise recombination with restoration of the wild type allele (data not shown). The failure to obtain an actual deletion mutant is a result of *rpoB* being an essential gene.

4. Discussion

Counterselectable markers are useful tools in molecular microbiology. We show here that expression of *E.coli galK* confers sensitivity to 2-DOG in fast and slow growing mycobacteria. Until now, *Bacillus subtilis sacB* has been the most commonly used counterselectable marker in mycobacteria. Although extremely useful, it has the disadvantage of a spontaneous mutation rate that is higher than the rate of rare recombination events necessitating laborious counterscreening to distinguish genetic recombination events from *sacB* inactivation.

The catalase gene *katG* from *M. tuberculosis* can be used as a counterselectable marker when cloned into fast growing mycobacteria such as *M. smegmatis*, because its affinity to

the drug isoniazid (INH) is approximately 20 times higher than that of the *katG* from *M. smegmatis* (Dubnau et al., 1996). However, the *katG* gene is large (2.2KB), and therefore less convenient to use in cloning. Also, this technique does not apply to slow growing mycobacteria, unless the *katG* gene was previously deleted, which would necessitate working with an INH resistant *M. tuberculosis* strain.

Mutations in the *rpsL* gene, encoding ribosomal protein S12, can confer resistance to streptomycin, and sometimes to other aminoglycosides as well (Bohman et al., 1984). Complementing such bacteria with *rpsL*⁺ copy restores dominant sensitivity to streptomycin, and was used for negative selection in several bacteria, including *S. pneumoniae* (Sung et al., 2001), *Streptomyces* (Hosted and Baltz, 1997) and *M. smegmatis* (Sander et al., 1995). However, as is the case for *katG*, the use of this method requires working with a clinically-relevant antibiotic resistant strain, in this case streptomycin.

The *E. coli galK* gene is only 1.3KB long, which makes it easier than *katG* or *sacB* for cloning and genetic manipulation. We show here that both in fast and slow growing mycobacteria, selection on 0.2% 2-DOG is highly effective with approximately one background colony for every 10⁵ CFU. For the preparation of deletion mutants in the 2 step allelic exchange method (Fig 5) we show that selection on 0.5% 2-DOG is at least as good, if not better, than use of 5% sucrose in selecting for precise second recombination (as measured by ratio of precise recombination to spontaneous 2-DOG or sucrose resistance). Most important, a strategy combining *sacB*/sucrose and *galK*/2-DOG (at 0.2%) was 100% effective in selecting for recombination events. A main drawback of the 2 step allelic exchange system using a single counterselection marker is a rate of spontaneous marker inactivation, leading for a need to re-screen for hygromycin sensitivity. The use of *galK* with *sacB* together eliminates the need for the counter screening on hygromycin, thus saving one week and considerable labor when working with *M. smegmatis*, and potentially at least 4–5 weeks of time and labor when working in *M. tuberculosis*. Using the *galK/sacB* system, 2-DOG/sucrose resistant colonies can be directly screened by Southern analysis, as our experiments indicate that nearly 100% of these colonies will be precise recombinants. A potential limitation of *galK* and 2-DOG use is its price – approximately 50 USD for 1 gram. However, given the strict selection, only a small number of plates needs to be used for every experiment, making the use of 2-DOG worthwhile, especially considering the time and labor saved.

In summary, we have documented the utility of *galK*/2-DOG as a counterselectable marker system in mycobacteria. This system further expands the available tools for genetic manipulation of mycobacteria.

Abbreviation

2-DOG	2-deoxy-galactose
PCR	Polymerase Chain Reaction
OADC	Oleic acid, Albumin, Dextrose, catalase
CFU	Colony Forming Units
WT	Wild Type
MOP	Mycobacterial Optimized Promoter
INH	Isoniazid
ligD	ligase D

galK	galactokinase
katG	catalase
rpsL	30S ribosomal subunit protein
rpoB	β subunit of the bacterial RNA polymerase
sacB	levan sucrose

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Figure 1.

Two of the plasmids used in this study are shown. Left: pDB77 has the *E.coli galK* gene cloned under the MOP promoter, and an *attP* site (but no integrase). Right: pDB88 contains *E. coli galK* expressed from the MOP promoter, *sacB*, *hygR*, and the 5' and 3' flanking regions of *ligD*. There is an *oriE*, but no *oriM*, preventing replication of the plasmid in mycobacteria. See methods for details of construction.

Figure 2.

The indicated number of MGM1959 (10^2 – 10^5 *M. smegmatis* with *mycgalK*) were plated on 7H10 plates (top row) or 7H10 with 0.2% 2-DOG (bottom row).

Figure 3.

A. MGM1970 (*M. smegmatis* with *E. coli galK*) and a control strain (*M. smegmatis* with pYUB412) were plated on a 7H10 plate (left), or 7H10 supplemented with 0.2% 2-DOG (right).

B. The indicated number of MGM1970 CFUs (10^2 – 10^7 colony forming units) were plated on 7H10 plates (upper panel) or 7H10 plates with 0.2% 2-DOG (lower panel).

Figure 4.

A. Approximately 1000 CFUs of MGM1970 were plated on each plate, with the concentration of 2-DOG escalating from 0 to 0.05, 0.1 and 0.2%. Pictures were taken after 72 hours of incubation.

B. Approximately 10^6 CFUs of MGM1971 (*M. tuberculosis* with *E. coli galK*) and *M. tuberculosis* Erdman WT were plated on a 7H10 plate with 0.2% 2-DOG.

Figure 5.

A: Map of the genomic region of *ligD* after a single recombination with pDB88 at the 3' flanking region of *ligD*. All of pDB88 is integrated into the genome. The restriction sites for NaeI and XmnI are shown, as are the predicted sizes of the restriction fragments, when probed by Southern blotting with the 5' flank of *ligD* (indicated by a gray rectangle labeled P in panels A and B). The strain is sensitive to sucrose and 2-DOG, but resistant to hygromycin.

B and **C** depict the two mechanisms of resolution of the tandem structure in A by recombination. The strains will be sucrose and 2-DOG resistant, and hygromycin sensitive. B is the result of recombination via the 3' flank of *ligD*, reverting the strain to WT, whereas

C is the result of recombination at the 5' flank of *ligD*, replacing *ligD* with the deletion allele. Restriction sites and predicted sizes on Southern blotting are shown.

D: This map depicts the genomic region in which no second precise recombination event occurred, but a spontaneous mutation or deletion inactivated both counter-selectable markers. *ligD* is not deleted, and the strain is sucrose/2-DOG resistant, but also hygromycin resistant. The predicted sizes on a Southern blot will be the same as in A.

Figure 6.

A. Southern blot of WT *M. smegmatis* (WT) or *M. smegmatis* with a single cross over at the 3' flanking region of *ligD* (intermediate; int). Genomic DNA was digested with XmnI. When probed with the 5' flanking region of *ligD*, WT genomic DNA gives a band size of 3.16kb, whereas the intermediate gives two bands of 3.16 and 10.1 kb. The additional band to the right is non-specific probe hybridization to the 1.6 kb marker band.

B. Southern analysis of recombination products after counterselection. Left to right: 1kb DNA ladder marker, WT, intermediate, the 7 hygromycin sensitive colonies counter-selected for a precise second recombination event on 0.5% 2-DOG, and 9 of the 39 hygromycin sensitive colonies selected on 2-DOG/sucrose. Genomic DNA was digested with NaeI, and the probe is the same as in A. WT yields a band of 3.84kb, intermediate yields 6.08 and 6.65kb, and deletion mutant is 1.52kb. The probe non-specifically binds to the 1.6kB band of the molecular weight marker.

Table 1

The observed frequency of hygromycin sensitive colonies (Hyg^{Sensitive}) to total colonies growing on the indicated counter selection agent is shown for two different genetic loci, *ligD* and *rpoB*.

Targeted gene	Counter selection agent	Hyg ^{Sensitive} /total colonies (precise second recombination)
<i>ligD</i> (two experiments)	0.5% 2-deoxy-galactose	7/51 (13.7%) 9/45 (20%)
	5% Sucrose	0/51 (<2%) 3/84 (3.5%)
	0.2% 2-deoxy-galactose + 5% Sucrose	39/39 (100%) 17/17 (100%)
<i>rpoB</i>	0.5% 2-deoxy-galactose	Not Done
	5% Sucrose	7/130 (5.4%)
	0.2% 2-deoxy-galactose + 5% Sucrose	75/76 (98.7%)