Journal of Experimental Microbiology and Immunology (JEMI) Vol. 13:79-82

Analysis and Comparison of β-galactosidase Production in *Escherichia coli* BW25993/Δ*lacI* and MG1655/Δ*lacI* Mutants During Conditions of Growth and Catabolite Repression

Bao R. Huo, Roger Jen, Petra Lolić and Derek To
Department of Microbiology & Immunology, UBC

In previous studies, successful knock-outs of the *lacI* gene in various *Escherichia coli* (*E. coli*) strains was created. Further testing of the MG1655/Δ*lacI* strain grown at 30˚C in M9 glycerol minimal media showed wild type basal levels of β-galactosidase production. Potential explanations for such a result could include faulty chromosomal gene alterations, metabolic alterations due to differences in growth phase, or media selection that introduced catabolite repression exclusively to the MG1655/Δ*lacI* strain. In this study, a qualitative inspection of growth of wild type and Δ*lacI* mutant strains of *E. coli* on various differential and selective plates was used to determine if the strains created in Beamish *et al's* study have a faulty chromosome insertion of the kanamycin selection cassette. Quantitative growth and β-galactosidase enzyme activity assays were performed to see if the bacterial growth phase and catabolite repression play a crucial role in the basal β-galactosidase production levels of the MG1655/Δ*lacI* mutant. Preliminary data revealed that all strains of Δ*lacI* mutants showed constitutive β-galactosidase production in the absence of an inducer, contrary to previous findings. The study revealed that catabolite repression and early growth up to four hours in relatively low bacterial density are not responsible for the reported wild type basal level production of β-galactosidase seen in the MG1655/Δ*lacI* mutant in the previous study.

The regulatory *lacI* gene codes for the LacI β-galactosidase-repressor. When it is expressed, it inhibits the transcription of *lacZ*, *lacY* and *lacA* genes (6). These genes are required for the metabolism of lactose in *E. coli* and are therefore been extensively studied. In Beamish *et al*'s study (1), successful disruption of the *lacI* gene was done using the Lambda Red Recombinase system in *E. coli* strains C29, BW25993, and MG1655. However, confirmation of the *lacI* deletion through β-galactosidase enzyme assays showed that MG1655/Δ*lacI* grown at 30˚C in M9 glycerol minimal media had an unexpected low, basal, wild type level of β-galactosidase enzyme activity. We suspected that the result was due to faulty chromosomal gene alteration (8), metabolic alterations due to the effects of metabolic waste products in the surrounding media (5), or media selection that introduces catabolite repression exclusive to the MG1655/Δ*lacI* strain (7).

Faulty chromosomal gene alteration involves the impartial deletion of the *lacI* gene (4). Since the Lambda Red Recombinase system applied in Beamish *et al*'s (1) study involved the replacement of the *lacI* gene with a kanamycin cassette, the authors suggested that perhaps the insertion of the kanamycin cassette into the *lacI* gene did not result in a complete deletion of the *lacI* gene. It has been observed before that some forms of residual *lacI* gene can still result in repression of β-galactosidase activity (3).

Catabolite repression is an alternate controlling mechanism that modifies and reduces the transcription level of β-galactosidase in the presence of a better carbon source such as glucose during growth (7). In the absence of glucose, cyclic AMP binds to the catabolite activator protein (CAP), which in turn allows the CAP to bind to the CAP promoter and assist in increasing expression of the *lac* operon (7).

It has been shown that the production of β-galactosidase in *E. coli* decreases as the metabolic by-products increase due to an increase in culture density (5). The cultures used in Beamish *et al*'s study (1) were not standardized to the same culture density. Therefore, the β-galactosidase activity data of the MG1655 mutant might have been representative of a culture grown in a media high in metabolic waste products whereas the BW25993 mutant may have been in a growth media with lower metabolic waste products. Such differences in media composition amongst the cultures might have played a role in the difference of β-galactosidase production seen.

This study was designed to examine and compare the production of β-galactosidase between BW25993/Δ*lacI* and MG1655/Δ*lacI* strains grown in the presence or absence of glucose over a short time period. To investigate catabolite repression, we selected BW25993 as the positive control strain and the MG1655 strain to test under three growth conditions. The growth conditions included M9 minimal glycerol media, M9 minimal glucose media, and M9 minimal glucose media with IPTG inducer. The presence of glucose as a carbon source was used to investigate the presence of catabolite repression on the *lac* operon. Enzyme activity assays were performed on the strains in each growth condition to quantify β-galactosidase activity. As well, the culture density was measured throughout different time points to investigate if culture density plays a crucial role in β-galactosidase production.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains BW25993/pKD46, BW25993/Δ*lacI*, MG1655/pKD46, MG1655/Δ*lacI*, C29/pKD46, C29/Δ*lacI* were obtained from the MICB 421 Bacterial Stock Collection (Department of Microbiology and Immunology, Unversity of British Columbia). Strains were streaked and grown on M9 minimal glycerol media agar plates (M9 salts: 0.05% NaCl, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 1% NH_4Cl; supplemented with and 0.8 mM $MgSO_4$, 1% (w/v) glycerol, 0.5% Bacto-Tryptone, and 0.25% Bacto-Yeast) at 42˚C. Growth at 42˚C cures pKD46 plasmids from the strains. For a detailed list of strain characteristics, please refer to Table 1 in Beamish *et al*'s paper (1).

Qualitative galactosidase enzyme activity test. *E. coli* strains were streak plated on the following three selective/differential plates to test for galactosidase activity: M9 minimal media agar plates supplemented with 20 ug/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), M9 plate with X-gal and 40 ug/mL kanamycin (Sigma, Catalogue # K4000), M9 plate with X-gal and 0.1 mM IPTG. Incubation time period was 18 hours at 37˚C. Strains used were pre-incubated in 42˚C to cure pKD46 plasmids before subjected to plate test.

Sampling and growth rate data for quantitative galactosidase enzyme activity test. *E. coli* BW25993 and MG1655 wild type (WT) and Δ*lacI* strains were grown in M9 minimal media broth overnight at 37˚C. Three M9 media (M9 supplemented with 1% glycerol, 1% glucose, or 1% glucose with 0.1 mM IPTG) were inoculated with the *E. coli* strains to an OD of 0.075 and incubated at 37˚C with shaking. Growth of the cultures in the media was recorded as OD_{460} using Spectronic 20 spectrophotometer on an hourly basis up till 4 hours. 200 µL samples of the culture at each time point were mixed with 100 µL of toluene in separate test tubes and vortexed vigorously for 20 seconds to permeabilize the bacteria and placed on ice.

Quantitative galactosidase enzyme activity test. Samples mixed with toluene on ice, collected during the growth rate experiment, were incubated in a 30˚C water bath for 5 minutes. To the warmed samples, 1.1 mL TM buffer and 0.2 mL of 5 mM ONPG were added and placed back into 30˚C water bath. When sufficient yellow colour developed in each sample, 2 mL of 0.6 M sodium carbonate was added. The time from the addition of ONPG till the addition of sodium carbonate was taken and used for the calculation of enzyme activity. Using Spectronic 20 spectrophotometer, the aqueous bottom phase from the test tubes was carefully aspirated and the absorbance read at 420 nm.

RESULTS

Confirmation of Δ*lacI* with kanamycin cassette. Observations in Table 1 confirm the Δ*lacI* genotype in the BW25993 and MG1655 strains. Blue bacterial growth seen for BW25993/Δ*lacI* and MG1655/Δ*lacI* strains show that β-galactosidase was expressed in a much higher level than the white growth observed for the wild type strains. The presence of blue colony growth when the cells were plated with IPTG, a known inducer of β-galactosidase production via the inactivation of LacI, was the positive control growth media, observed as functional β-galactosidase protein expression and activity. Growth on kanamycin was consistent with the presence of a kanamycin resistance gene inserted into the *lacI* gene.

TABLE 1. Qualitative confirmation of successful Δ*lacI* in *E. coli* strains. Strains grown on selective/differential plates with different supplements at 37ºC for 18 hrs (colors of the colonies are listed)

***E. coli* strains**	**M9 minimal media supplement**		
	X-gal	**X-gal + IPTG**	**X-gal + Kanamycin**
BW25993 WT	White	Blue	No growth
BW25993Δ*lacI*	Blue	Blue	Blue
MG1655 WT	White	Blue	No growth
MG1655Δ*lacI*	Blue	Blue	Blue

β-galactosidase activity in Δ*lacI* strains. A significant increase in β-galactosidase enzyme activity was observed when any form of alleviation of LacI repression was present. As shown in Table 1 and Figure 1, the β-galactosidase activity levels found in the wild type strains grown in media lacking IPTG were significantly lower than the enzyme activities observed for strains grown in the presence of IPTG or the Δ*lacI* mutants.

Growth phase and β-galactosidase activity. After growth curve analysis was performed on all *E. coli* strains growing in the different media conditions (data not shown), there was no apparent correlation between the enzyme production of β-galactosidase and the growth phase in which the *E. coli* strains were in. In most cases where glucose was used, the beginning of stationary phases was reached by 3 hours for most strains, while glycerol media growth showed slower entry into stationary phase.

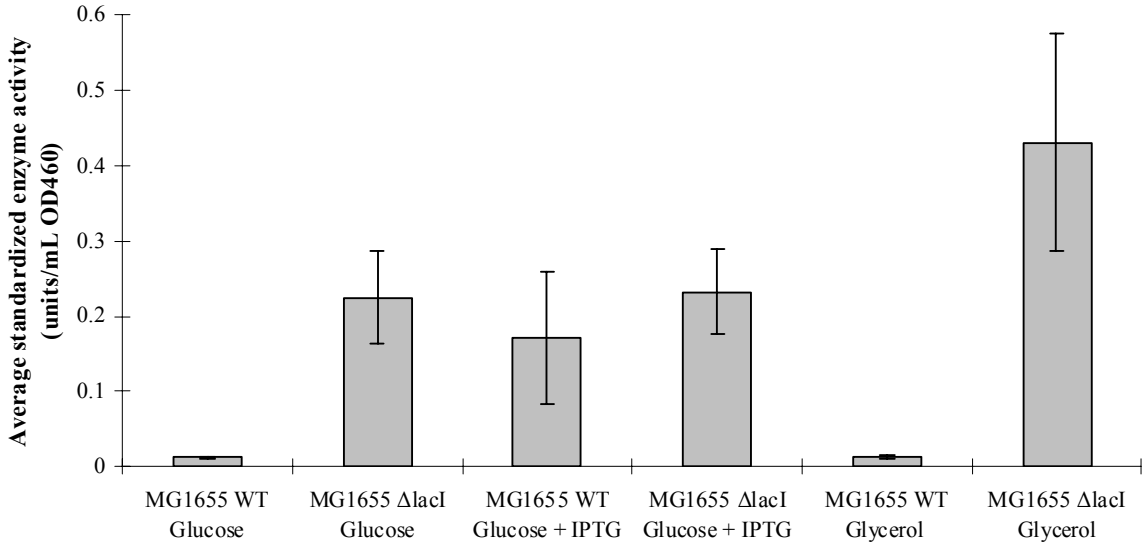

FIG. 1. Average fully-induced-culture-density normalized β-galactosidase enzyme activity of MG1655 WT/Δ*lacI* strains show catabolite repression when grown in the presence of glucose.

Repression of β-galatcosidase activity. The repression effect seemed to be only present in the absence of LacI repression of β-galactosidase production (either the Δ*lacI* mutants or wild type grown in IPTG) and when cultures are grown with glucose. As seen in Figure 1, the basal levels of β-galatcosidase production in MG1655 WT grown in glucose and glycerol supplemented media show no differences. When LacI repression was alleviated, such is the case in the Δ*lacI* mutants and growth in the presence of IPTG in wild type strains of MG1655, a trend can be seen in which the presence of glucose seems to repress the average β-galactosidase activity. However, the only significant difference seen is in the enzyme activity reading of MG1655 wild type strain in glucose and IPTG supplemented media compared to MG1655Δ*lacI* strain grown in glycerol-supplemented media. This observed trend was also seen for the BW25993 WT/Δ*lacI* strains, however, enzyme activity levels were halved in value compared to the respective MG1655 strains and treatments.

DISCUSSION

In testing to determine whether the MG1655/Δ*lacI* strain had a functional *lacI* gene, the results from Table 1 confirmed the complete deletion of the *lacI* gene. The growth of the wild type strains of BW25993 and MG1655 on M9 minimal media with X-gal led to the development of white colonies, as expected, due to the presence of LacI repressor inhibiting the expression of the *lac* operon. When the mutant Δ*lacI* strains of BW25993 and MG1655 were plated on X-gal, blue colonies developed which suggested that the strains were indeed *lacI* knockouts. If some *lacI* expression were still present due to partial deletion, the colonies would become bluer in color as the IPTG inhibits LacI from binding the *lac* operon. Further evidence to support this conclusion came from our quantitative analysis of β-galactosidase expression level (Fig. 1). It seemed there was no significant difference in β-galactosidase activity level between strains grown on M9 glucose minimal media and M9 glucose minimal media with IPTG for both MG1655/Δ*lacI* and BW25993/Δ*lacI* strains (FIG. 1). This unresponsiveness to IPTG suggested that our first hypothesis of partial deletion was not a factor in the unexpected result of low β-galactosidase production in the MG1655/Δ*lacI* strain observed in Beamish *et al*'s paper (1).

In the second part of our investigation, we looked into catabolite repression as a possible explanation for the results reported in Beamish *et al*'s paper (1). From Figure 1, we saw that the measured β-galactosidase activity in MG1655 WT grown on M9 minimal glucose media with IPTG was significantly lower than growth of the MG1655/Δ*lacI* on the M9 minimal glycerol media. Glycerol was used as an alternate carbon source for the bacteria and since it does not affect cAMP levels, it is considered as a low catabolite repression molecule (2). This observation suggested that, indeed, catabolite repression effect was present in the MG1655/Δ*lacI* strain. However, there was a trend that is visible, in which BW25993/Δ*lacI* grown on M9 glucose minimal media showed a decrease in β-galactosidase activity compared to BW25993/Δ*lacI* grown in glycerol. This

suggest that catabolite repression was not the underlying mechanism that was causing the decreased β-galactosidase activity reported in Beamish *et al*'s paper (1) since catabolite repression seemed to also be present to a similar extent in the BW25993 strain.

Another speculation for the basal level expression of β-galactosidase in the MG1655/Δ*lacI* strain of Beamish *et al*'s experiment (1) was the increase in metabolic waste products in the growth media. According to their protocol (1), a 1:15 dilution was performed on the overnight cultures of both MG1655 and BW25993 strains prior to inoculation of the fresh M9 media. In comparison, the inoculation performed in this study was approximately 1:50 dilution with a final growth culture of 2.0-3.0 OD_{460} units. The enzyme assay in Beamish *et al*'s paper (1) may not have been standardized properly since the 1:15 dilution inoculation may have caused a higher culture density in the growth flasks for the MG1655/Δ*lacI* strain. Thus, it may have been that the MG1655/Δ*lacI* strain was in a media with a less diluted nutrient concentration and higher waste product concentration. The higher waste product concentration may have inhibited β-galactosidase production (5) compared to other strains of MG1655 or BW25993.

Both qualitative and quantitative analysis has confirmed that MG1655/Δ*lacI* does exhibit higher β-galactosidase expression compared to the control MG1655 WT and BW25993 WT strains, contrary to Beamish *et al*'s findings. MG1655/Δ*lacI* does seem to exhibit significant catabolite repression, however the trend is apparent in the control BW25993/Δ*lacI* strain as well. The culture density that the bacteria were allowed to grow in this study was not high enough that the results can be comparable to those used in Beamish *et al*'s study (1). In conclusion, this study verifies that the Lambda Red Recombinase experiment utilized in Beamish *et al*'s paper (1) successfully created a MG1655/Δ*lacI* mutant. Catabolite repression does not seem to be the underlying mechanism that was inhibiting the production of β-galactosidase in the MG1655/Δ*lacI* strain. We speculate that the basal expression of β-galactosidase in MG1655/Δ*lacI* strain may have been due to extremely high culture density beyond 2.0-3.0 OD_{460} units. This factor affecting β-galactosidase expression will allow for future research focusing on the expression levels of β-galactosidase in *E. coli* in regards to long term culture growth.

FUTURE EXPERIMENTS

Further investigation into the control of levels of β-galactosidase enzyme levels between the MG1655 and BW25993 WT and Δ*lacI E. coli* strains should be pursued. We speculate that the underlying mechanism seen in the overall reduced levels of β-galactosidase in the BW25993 strain may be due to alternate intrinsic factors. An analysis of the promoters of MG1655 and BW25993 for the *lac* operon should be investigated since it is possible that the promoter of the *lac* operon may have been changed due to the transformation and insertion of the kanamycin cassette upstream in the *lacI* region. Sequencing the promoter region might reveal minor changes that would explain the difference in β-galactosidase production between MG1655 and BW25993.

ACKNOWLEDGEMENTS

This research was supported by the Department of Microbiology and Immunology at the University of British Columbia.

The authors would like to thank Dr. William Ramey and Jessica Labonté for their guidance and support throughout the project. We would also like to thank the Wesbrook building laboratory staff for helping with providing a safe and prepared environment for us to work in.

REFERENCES

1. **Beamish, L., R. Greenwood, K. Petty, and E. Preston**. 2008. 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.
2. **Bettenbrock, K., S. Fischer, A. Kremling, K. Jahreis, T. Sauter, and E.D. Gilles**. 2006. A quantitative approach to catabolite repression in *Escherichia coli*. J. Biol. Chem. **281**:2578-2584.
3. **Boer, J.G., and B.W. Glickman.** 1998**.** The *lacI* gene as a target for mutation in transgenic rodents and *Escherichia coli*. Genetics. **148**:1441-1451.
4. **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.
5. **Kurland, C.G. and H. Dong**. 2003. Bacterial growth inhibition by overproduction of protein. Mol. Microbiol. **21**:1-4.
6. **Santillán, M., and M.C. Mackey**. 2008. Quantitative approaches to the study of bistability in the *lac* operon of *Escherichia coli*. J. R. Soc. Interface. **5:**29–39.
7. **Wanner, B. L., R. Kodair, and F.C. Neidhardt**. 1978. Regulation of *lac* operon expression: reappraisal of the theory of catabolite repression. J. Bacteriol. **136**:947-954.
8. **Woo, A**. 2004. Characterizing a lambda red recombinase induced presumptive partial deletion of *lacI* gene in *Escherichia coli* C29. J. Exp. Microbiol. Immunol. **6**:1-8.