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Genetics Education

Innovations in Teaching and Learning Genetics

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Using Student-Generated UV-Induced *Escherichia coli* Mutants in a Directed Inquiry Undergraduate Genetics Laboratory

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ABSTRACT

We report a thematic sequence of directed inquiry-based labs taking students from bacterial mutagenesis and phenotypic identification of their own self-created mutant, through identification of mutated genes by biochemical testing, to verification of mutant alleles by complementation, and finally to mutant allele characterization by DNA sequence analysis. The lab utilizes UV mutagenesis with wild-type *Escherichia coli* and a UV-sensitive isogenic derivative optimized for undergraduate use. The labs take advantage of the simplicity of *E. coli* in a realistic genetic investigation using safe UV irradiation methods for creation and characterization of novel mutants. Assessment data collected over three offerings of the course suggest that the labs, which combine original investigation in a scientifically realistic intellectual environment with learned techniques and concepts, were instrumental in improving students' learning in a number of areas. These include the development of critical thinking skills and understanding of concepts and methods. Student responses also suggest the labs were helpful in improving students' understanding of the scientific process as a rational series of experimental investigations and awareness of the interdisciplinary nature of scientific inquiry.

As scientist educators we strive to involve students in the process of active, engaged scientific inquiry, where they can benefit in a number of ways from involvement in projects where they are “doing real science” as opposed to performing scripted “cookbook” activities. These include (1) gaining an understanding of how scientific studies are conducted, (2) confirming that such studies yield credible results, and (3) seeing how engaging scientific discovery can be. The report of the Committee on Undergraduate Biology Education to Prepare Scientists for the 21st Century underscored the significance of involving and inspiring students through active learning to better prepare them as future scientists and “give them an enduring sense of the power and beauty of creative inquiry” (NATIONAL RESEARCH COUNCIL 2003, p. 2). Findings also emphasized the

importance of interdisciplinary research experiences and using “examples of research showing that science consists of unanswered questions” to “intrigue and inspire students to probe problems in depth” (NATIONAL RESEARCH COUNCIL 2003, p. 3).

The integration of discovery-based exercises into undergraduate genetics laboratory curricula poses many challenges. These include budget limitations, performing activities within prescribed laboratory period constraints, and student mastery of techniques as a prerequisite to actual experimentation. However, the rewards of active and discovery-based approaches are substantial (HANDELSMAN *et al.* 2004). As compared to traditional approaches, active-learning exercises can result in increased knowledge retention, student confidence, enthusiasm, and satisfaction (WYCKOFF 2001; SMITH *et al.* 2005). Marcus and Hughes developed inquiry-based genetics lab exercises using *Drosophila melanogaster* strains harboring *P*-element transposon insertions in novel recombination mapping activities (MARCUS and HUGHES 2009); and while student re-

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.110.120147/DC1>.

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sponses to the labs were variable, most students were excited and inspired by doing real experimental science.

To improve the undergraduate biology curriculum at Trinity University through discovery-based and active-learning exercises, we developed labs in which students create and analyze novel lactose metabolism mutations in *Escherichia coli*. The labs intellectually and experimentally link learned techniques to original, unscripted investigations over an 8-week period. Instead of using organisms with known genotypes, students use safe UV mutagenesis methods and facile screens to create their own strains, thereby gaining a sense of project ownership. A logical sequence of mutant characterization follows, which includes enzymatic and substrate transport assays, plasmid transformation-based complementation, and amplification and nucleotide sequencing of mutant alleles. The activity culminates in comparisons of mutant and wild-type sequences to facilitate an understanding of mutations and their role in altered protein structures, connecting phenotype to genotype.

It was our hope that this active-learning investigative approach, using activities without scripted answers, would aid student understanding of genetics concepts. Additionally, students may see improved understanding of the process of scientific inquiry and linkage between the larger picture of scientific investigation and stepwise accumulation of findings leading toward scientific discovery. Assessment data collected following three consecutive years of teaching the sequence suggest that the labs are effective in developing student understanding of genetics, scientific inquiry, and interdisciplinary crosstalk (*e.g.*, between biochemistry, genetics, and chemistry) in the process of scientific investigation.

MATERIALS AND METHODS

E. coli was chosen because (i) handling and maintenance are inexpensive and require little training; (ii) *E. coli* grows rapidly, with doubling times of ~30 min in rich media, allowing completion of exercises within a day or two; (iii) many individuals can be analyzed (at appropriate densities, mutant phenotypes can be readily distinguished from among hundreds of surrounding colonies in a single dish); (iv) mutagenesis of the haploid genome readily yields interesting mutants; (v) plasmid complementation is done in a day; and (vi) mutant allele PCR amplification and nucleotide sequencing allow comparative analysis with wild-type sequences.

We focused on the *lac* operon because it is usually covered in introductory genetics courses and can serve as a “Rosetta Stone” connecting textbook to lab. Available literature on *lac* operon structure–function relationships makes it a logical place to expose students to gene structure, function, regulation, and protein structure.

The exercises cover 8 weeks of a 12-week sophomore genetics laboratory, leaving time to repeat experiments if necessary. At the end, students’ findings are reported in a research paper. Labs are taught to three or four sections of 16 students (depending on enrollments); each section meets weekly for ~3 hr. Students work in pairs and are required to complete a two-semester introductory biology course before

enrolling in the course. Students learn pipette use in the introductory course and learn bacteriology skills in the genetics lab. Protocols and notes are available (supporting information, [File S1](#)), and all strains and plasmids are available upon request.

Bacterial strains and plasmids: *E. coli* K12 strains were cultured at 37° in Luria–Bertani (LB) broth or on LB or MacConkey agar medium with added kanamycin (Km, 50 µg/ml), tetracycline (15 µg/ml), or ampicillin (100 µg/ml) where appropriate. UV mutagenesis was done using strains W3110 [F^- , λ^- , $IN(rrnD-rrnE)1$] and an isogenic *uvrA-phr* mutant, constructed using standard P1 transduction with strains JW0698 [$\Delta(araD-araB)567 \Delta lacZ4787(::rrnB-3) \Delta phr-758::Km \lambda^- rph-1 \Delta(rhaD-rhaB)568 hsdR514$] and N3055 [$\lambda^- IN(rrnD-rrnE)1 uvrA277::Tn10$]. *lacY* and *lacZ* complementing plasmids were prepared from the Clarke and Carbon collection (CLARKE and CARBON 1976) and using standard cloning and PCR methods ([File S1](#)).

Week 1. Handling of bacterial cultures and dilution plating: Students are introduced to the philosophy and scope of the labs and are instructed that they will generate their own mutants and characterize them in detail, from phenotypic analysis to nucleotide sequence analysis. We emphasize they will perform a real genetics experiment in the sense that neither they nor the instructor have *a priori* knowledge of the properties of mutants they will create and they need to think about how geneticists methodically approach problems using various techniques in logical sequences, each bringing the researcher closer to the answer. Students learn microbiological techniques for use throughout the semester, including aseptic technique, serial dilution, and streaking cultures.

Week 2. UV mutagenesis of wild-type *E. coli* strain W3110 and UV-sensitive strain W3110-*uvrA-phrA6*: This lab introduces selections *vs.* screens. Mutant generation as a powerful genetics tool is discussed with the connection between phenotype and nucleotide sequence level genotypic differences. DNA damage, mutation, and repair provide the rationale for using isogenic strains. A genetic *screen* for defects in lactose fermentation on MacConkey agar is contrasted with a *selection* for antibiotic resistance in antibiotic-sensitive bacterial populations they see in complementation assays.

UV mutagenesis was selected over more efficient chemical mutagens for safety reasons; it was selected over transposition-based methods because it produces broader mutant classes with unique and subtle properties, for example, temperature sensitivity or alterations in catalysis due to amino acid substitutions.

The class is divided into two teams for mutagenesis. Since this lab requires organization and attention to detail, one student from the lab section is chosen to organize the activity. She or he assigns students to timing of UV exposures of cultures and plating of time points, etc. One team irradiates the wild-type strain, while the second team irradiates the UV repair mutant, using cell suspensions exposed to a UV lamp for four times at each of four distances, for a total of 16 different treatments. Aliquots (1.5 ml) of cells in cold 0.1 M $MgSO_4$ are dispensed into 35-mm petri dishes and irradiated using a 15-W Sylvania G15T8 germicidal lamp with irradiance and fluence rates of 600 mW/m² and 1.3 µmol/m²/sec, respectively (SHINKLE *et al.* 2005). Students are protected by hanging the lamp behind a black curtain. Dishes are placed on a tray and the lids are removed immediately before being placed behind the curtain. It is imperative that lids are removed to permit UV exposure; it is also critical that the dishes (not the lids) are labeled before irradiation to keep track of strains and treatments. Dishes are removed from the UV source at appropriate time points and teams plate serial dilutions of each strain \times distance \times time on MacConkey agar

to determine survival frequencies. Nonirradiated cells are plated out to provide initial culture densities; these data provide survival frequencies between the strains and illustrate the deleterious effects of UV exposure and the importance of UV-induced lesion repair activities.

Suspensions of the mutagenized UV repair mutant are plated to identify mutants; this activity needs to be done only with the UV repair mutant, as little mutagenesis occurs with the parent strain W3110. Optimal exposures using our lamp setup are described in File S1; for UV irradiation experiments, survival frequencies in the range of 0.1–1% result in the highest mutant recovery frequencies (MILLER 1972).

Students inspect plates for rare Lac⁻ colonies among many red Lac⁺ colonies. In optimal irradiation ranges, 2–10 lactose-nonfermenting mutants can be found on plates containing ~500 colonies. Putative mutants are streaked onto MacConkey-Km plates to obtain single colonies and are maintained on plates for the duration of the lab; we encourage students to identify several mutants, since they may find false positives or strains with mutations lying outside of *lacY* or *lacZ* (e.g., *lacI* or *crp*; see RESULTS AND DISCUSSION). Depending on colony density, mutant genotype stability, and colony picking skills, students may streak more than once to ensure they have stable single-colony Lac⁻ isolates before proceeding to subsequent experiments. It is useful here to build extra time into the schedule to allow students to establish stable Lac⁻ strains before proceeding to the next exercise.

Irradiated cultures can also be screened for other mutants, e.g., auxotrophs or temperature-sensitive mutants, and/or exercises can be modified to more open-ended approaches where students are asked to devise their own screens/selections.

Week 3. The β -galactosidase assay for biochemical characterization: *lac* operon components are presented, including *lacY*, encoding the proton symport membrane protein required for lactose uptake; *lacZ*, encoding β -galactosidase, required for hydrolytic cleavage of lactose into glucose and galactose; and *lacI*, encoding the lactose repressor, which prevents *lac* expression in the absence of lactose.

The qualitative β -galactosidase assay is used to determine whether cells produce functional *lacZ* gene products by assaying cell suspensions for the appearance of *o*-nitrophenol, the yellow hydrolysis product resulting from cleavage of *o*-nitrophenyl- β -D-galactopyranoside (ONPG). The assay also demonstrates that *lac* expression is inducible with the inclusion of isopropyl- β -D-thiogalactopyranoside (IPTG) in cultures. Instructors can discuss reaction chemistry involved in glycosidic bond cleavage, particularly regarding reactive amino acids in the protein, as this can later be tied into *lacZ* mutant analysis at the nucleotide sequencing stage.

It is apparent to students that Lac⁻ phenotypes could be attributed to mutations affecting *lacZ* gene product activity or expression. It is less obvious that Lac⁻ mutants may possess defects in LacY: such mutants fail to produce colored products from ONPG because ONPG cannot enter the cell. *lacY* and *lacZ* mutants can be distinguished using whole intact cells and cells whose membranes have been permeabilized with chloroform. *lacZ* mutants yield a negative reaction using whole and permeabilized cells, whereas *lacY* mutants yield negative results in whole cells but positive results using permeabilized cells. Additionally, LacY is arguably the best understood proton symport protein (SMIRNOVA *et al.* 2008; GUAN and KABACK 2009; ZHOU *et al.* 2009; LIU *et al.* 2010), and structural relationships can be drawn between wild-type and mutant LacY following sequencing of mutant *lacY* alleles. The majority of mutants identified by students carry mutations in *lacZ* or *lacY*, but alternative outcomes are possible (see RESULTS AND DISCUSSION).

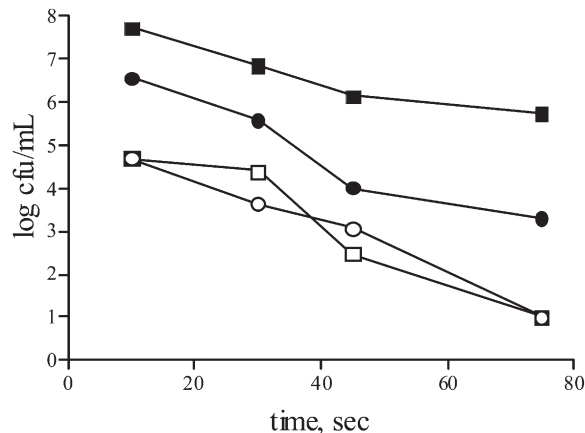


FIGURE 1.—Student-generated survival curves of *Escherichia coli* W3110 wild type (solid symbols) and repair mutant following UV irradiation. Exponential phase cultures were pelleted by centrifugation and resuspended in cold 0.1 M MgSO₄ and cell suspensions were irradiated for the indicated times at distances of 45 inches (■, wild type; □, mutant,) and 25 inches (●, wild type; ○, mutant,). Following irradiation, cells were plated on MacConkey agar and enumerated following overnight growth at 37°.

After completing this lab, students have biochemical evidence supporting predictions of what type of mutant they have and are ready to test these predictions using complementation analysis with plasmids carrying wild-type *lac* alleles.

Week 4. Plasmid extraction: Students are introduced to complementation and shown how mutant phenotypes could be attributed to specific gene defects by introducing wild-type alleles into mutants to see if the wild-type phenotype is restored. Students are given strains carrying *lacZ*⁺ and *lacY*⁺ plasmids and control vector pBR322, and they perform plasmid extractions. We use alkaline lysis for this but kits are also available.

Week 5. Agarose gel electrophoresis, spectroscopy, and complementation of Lac⁻ mutants: Agarose gel electrophoresis and UV spectroscopy are introduced for nucleic acid visualization and analysis using spectrophotometry. Students prepare competent cells of their mutants and control *lacZ* and *lacY* mutants for plasmid transformation (CHUNG *et al.* 1989) and complementation results are typically straightforward. If a Lac⁺ phenotype is not restored to mutants carrying *lacZ*⁺ or *lacY*⁺ plasmids, students can perform transformation using plasmids carrying *lacI* and *crp* (see RESULTS AND DISCUSSION).

Week 6. PCR amplification of mutant alleles from Lac⁻ mutants: Students use PCR to amplify alleles from DNA of mutants and control sequences from wild-type parent DNA. Primer sequences and PCR protocols are given in File S1.

Week 7. Agarose gel electrophoresis and DNA sequencing of PCR products: Students perform agarose gel electrophoresis to assess the success and specificity of amplification of mutant and wild-type control alleles. We have found it useful to build extra time into the schedule to troubleshoot and/or repeat failed PCR assays. Over the course of the week, PCR products are sent to the University of Texas (Austin, TX) for sequencing.

Week 8. Analysis of DNA sequencing results: The full scope and value of the lab are realized in the eighth week when students receive electropherogram and DNA sequence text files for comparison with wild-type sequences. A fundamental connection is established between phenotypic defects of mutants with genotypic alterations reflected in altered allele

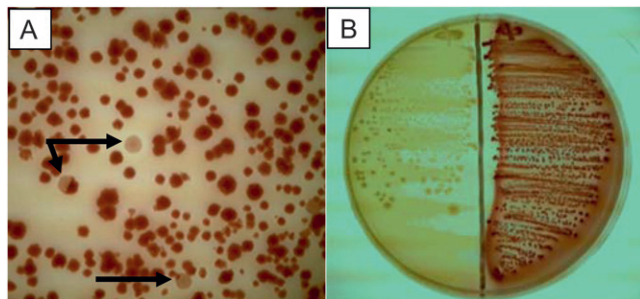


FIGURE 2.—Screen and single-colony isolation of Lac^- mutants on MacConkey agar following irradiation. Suspensions of repair mutant in 0.1 M $MgSO_4$ were irradiated for varying times and distances as described (File S1). Following outgrowth and segregation in LB medium, cells were plated on MacConkey agar and incubated overnight at 37°. (A) Mutagenized cells on MacConkey medium. Lac^- mutants are indicated with black arrows. (B) Comparison of Lac^- mutant (left) and wild-type Lac^+ parent (right) on MacConkey medium.

sequences. Students construct alignments of mutant and wild-type sequences to identify sequence changes.

RESULTS AND DISCUSSION

Experimental results: The mutagenesis protocol is standard but utilizes a DNA repair mutant we constructed for the lab; and in combination with the parent strain, students can directly compare the effects of irradiation at different distances and times within and between the two strains (Figure 1). The MacConkey screen permits visualization of white Lac^- colonies amid red Lac^+ colonies (Figure 2), although false positives will appear on plates. In particular, we see Lac^+ “fish eye” morphologies and false positives accompanied by the production of elevated levels of secreted polysaccharides, which initially appear Lac^- due to abundant whitish extracellular material. Students should cast wide nets and spend time outside of normal lab times to obtain *bona fide* Lac^- derivatives, although the initial mutant identification activity is typically the only time students need to be in the lab outside of the normal lab schedule.

The β -galactosidase assay provides an indication of the genetic basis underlying mutant phenotypes (Figure 3): $lacZ$ mutant extracts will not produce a yellow reaction product, whereas yellow reactions indicate functional $LacZ$. Nonpermeabilized $lacY$ mutant extracts will not turn yellow over short time courses, whereas chloroform-permeabilized $lacY$ extracts turn yellow within minutes.

Plasmid extraction and transformation are straightforward and connect complementation and cell biochemistry data; after this experiment, students can usually conclude whether they have isolated $lacZ$ or $lacY$ mutants.

As mentioned previously, students may isolate strains with mutations in genes other than $lacY$ or $lacZ$. Among

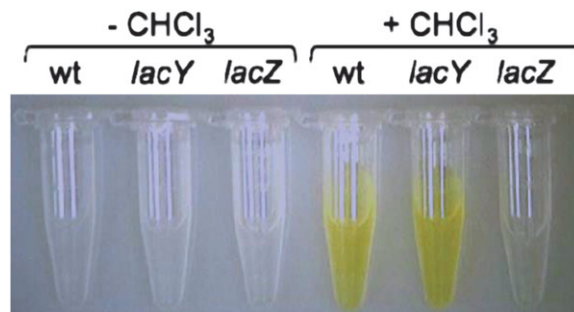


FIGURE 3.—Differentiation of $lacZ$ and $lacY$ mutants using a qualitative β -galactosidase assay. Cultures are grown in LB medium with IPTG for 2–3 hr before assaying cell extracts (File S1). β -galactosidase-dependent production of *o*-nitrophenol in chloroform-permeabilized cells (right, + $CHCl_3$) allows discrimination of $lacY$ mutants from $lacZ$ mutants. wt, wild-type Lac^+ *E. coli*; $lacY$, $lacY$ lactose transport mutant; $lacZ$, $lacZ$ β -galactosidase mutant. Over the short reaction time course shown (2–4 min), no activity is observed in intact cells that have not been permeabilized (left, $-CHCl_3$).

alternative Lac^- isolates, one might find (i) mutations in $lacI$, (ii) mutations in lac promoter or sequences bound by $LacI$, or (iii) mutations involved in CRP-dependent catabolite control. The MacConkey screen is not suitable for identification of $lacI$ null alleles that do not bind lac operator DNA: mutants of this type are typically Lac^+ on MacConkey medium and evade detection. However, one may generate $lacI$ alleles that bind lac operator DNA but that do not bind lactose or IPTG (ROMANUKA *et al.* 2009), and these mutants could appear Lac^- or weakly Lac^+ using the MacConkey screen. We suspect they would have variable β -galactosidase activities depending on their DNA binding kinetics but predict that β -galactosidase activities with these mutants would be identical regardless of whether they were grown in the presence or absence of IPTG. A $lacI^+$ plasmid is available for use although results may vary depending on DNA binding properties of the mutant gene product and dimerization interactions between itself and wild-type $LacI$.

Mutations could occur in *crp*, encoding the cyclic-AMP receptor protein (CRP or CAP), since efficient lac transcription requires RNA polymerase–CRP interactions (SHARMA *et al.* 2009). *crp* mutations could yield Lac^- mutants, and although we have not needed to use *crp^+* plasmids these are available. While picking multiple mutants increases the odds of recovering $lacZ$ or $lacY$ mutants, the less straightforward characterization of a mutant yielding atypical β -galactosidase results may be an appropriate project for a particularly gifted, invested, or enthusiastic student.

Amplification products are sometimes larger than expected due to Tn10 insertions (Figure 4). The *uvrA* allele used for construction of the mutagenesis strain carries a Tn10 insertion, and while we did not initially intend for students to find transposon insertion mutants, the unexpected discovery of insertions serves our initial aims well: to have students discover something in

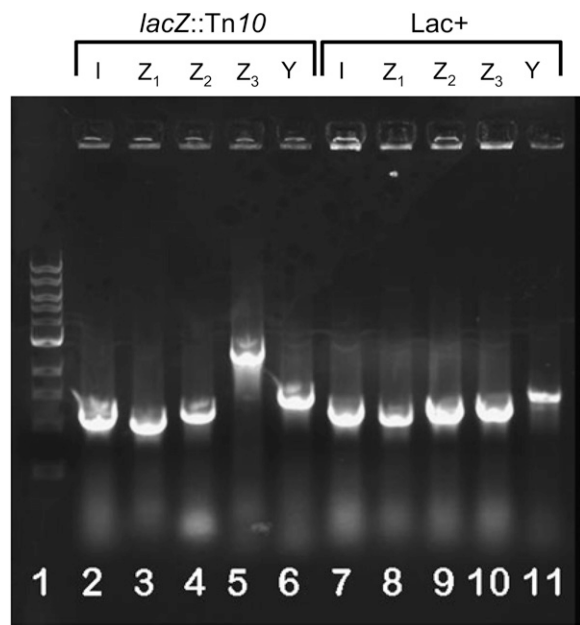


FIGURE 4.—Gel electrophoresis of *lac* allele amplification products from genomic DNA of *lacZ* Tn10 insertion mutant (lanes 2–6) and *Lac*⁺ parent (lanes 7–11). Lane 1, kilobase ladder; lanes 2 and 7, *lacI* amplification products; lanes 3 and 8, *lacZ* region 1 products; lanes 4 and 9, *lacZ* region 2 products; lanes 5 and 10, *lacZ* region 3 products; lanes 6 and 11, *lacY* products. Due to its larger size, *lacZ* is amplified and sequenced as three separate ~1-kb fragments; extraction and amplification methods are described in File S1.

an unscripted investigation and to feel engaged in and excited about the science.

Analysis of alleles reveals a variety of alterations (Figure 5). Close examination of 15 *lacY* mutant sequences revealed the following: 8 carried Tn10 insertions in three different sites; 1 carried an indel of >1.5 kb that extended beyond the *lacY* coding sequence; 1 carried both an indel and a missense substitution; 1 carried a frameshift mutation resulting from a single-nucleotide indel; and 4 were incomplete, likely resulting from low-quality templates following PCR amplification and cleanup. We have found that sequence quality is directly affected by template quality and care must be taken to ensure that PCR products are adequately cleaned up prior to sequencing.

To date we have concluded the lab with sequence analysis, although it may be broadened to include protein analysis. High-resolution structures of LacY, LacZ, and LacI could be used as templates to generate predictive structures, using homology modeling applications (e.g. SWISS-MODEL or 3Djigsaw) to explore how mutant alleles yield defective proteins.

Student evaluation of experience: Our goal was to use guided inquiry labs to facilitate understanding of the scientific approach to problem solving and critical thinking skills. In addition to learning genetics concepts, we wanted students to appreciate the importance of “interdisciplinarity” (e.g., genetics, biochemistry,

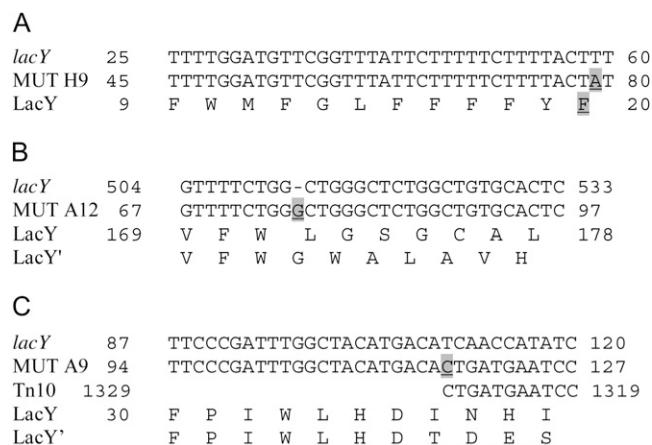


FIGURE 5.—Partial alignment of representative *lacY* mutant types with wild-type *E. coli* W3110 *lacY* sequences. Wild-type *lacY* sequence (*lacY*) is given at the top of each panel. (A) T → A transversion mutation (shaded and underlined) in mutant MUT H9 sequence found at nt position 59 with respect to wild-type *lacY* translation start, resulting in F59Y substitution in LacY permease. Altered phenylalanine residue (shaded and underlined in wild-type LacY sequence) occurs in LacY transmembrane domain 1 (MIRZA *et al.* 2006). (B) Frameshift mutation resulting from G insertion (shaded and underlined) in mutant MUT A12 at nt position 492 relative to *lacY* translation start. Mutation results in LacY derivative (LacY') with altered amino acid sequence beginning at residue 172, in transmembrane helix 6 (MIRZA *et al.* 2006). (C) Tn10 insertion in mutant MUT A9 at nt position 109 relative to wild-type *lacY* translation start. The first nucleotide of Tn10 insertion is shaded and underlined. Tn10 sequence is given as reverse complement of GenBank accession AY319289. The first four residues of altered peptide sequence (LacY') are underlined and occur in the N-terminal periplasmic domain (MIRZA *et al.* 2006).

etc.) in problem solving, as increasing interdisciplinary content is beneficial to learning and development (NATIONAL RESEARCH COUNCIL 2003). To assess these outcomes, surveys were administered over 3 years on the basis of an instrument reported by SLEISTER (2007). Survey questions used a five-point scale to assess perceptions of what students gained from the labs (Q1–Q4) and to compare the value of the directed inquiry approach to that of traditional labs (Q5–Q9) (Table 1). Traditional labs previously taken by students would include, e.g., introductory chemistry laboratory courses utilizing scripted activities with known outcomes.

Compared to before their participation, after taking the lab students agreed they had noted improvements in all areas queried, including understanding of scientific concepts (Q1, $\mu \geq 4$ over all 3 years) and the process of scientific inquiry itself (Q2, $\mu \geq 3.8$ over all 3 years). Students also perceived improvements in critical thinking abilities (Q3, $\mu \geq 3.7$ over all 3 years) and seeing connections between individual experiments (Q4, $\mu \geq 4.1$ over all 3 years). Additionally, mean values for Q1–Q3 increased over the assessment period, while the mean value for Q4 over survey year 3 was also higher relative to survey year 1.

TABLE 1
Assessment questions and responses

Think about your abilities at the beginning *vs.* the end of the semester. Using the scale 1–5, please indicate the level to which you agree with the following statements: 5, strongly agree; 4, agree; 3, neutral; 2, disagree; 1, strongly disagree. As compared with before participating in the genetics lab, after taking the genetics lab I noticed an improvement in the following:

	<i>F'</i> 07 (<i>N</i> = 36)	<i>F'</i> 08 (<i>N</i> = 29)	<i>F'</i> 09 (<i>N</i> = 37)
1. My understanding of technical/scientific concepts (<i>e.g.</i> , PCR, molecular cloning).	4.03 ± 0.91	4.21 ± 0.56	4.32 ± 0.75
2. My understanding of how science is done (<i>i.e.</i> , the range of activities from asking a biological question to conducting an experiment and interpreting data).	3.81 ± 0.86	4.00 ± 0.76	4.05 ± 0.70
3. My ability to think critically.	3.75 ± 0.91	4.17 ± 0.60	4.24 ± 0.72
4. My ability to make connections between individual experiments (<i>e.g.</i> , the “big picture” of the project).	4.14 ± 0.93	4.07 ± 0.70	4.46 ± 0.73

Have you taken a traditional science laboratory course? If you answered “yes” to the previous question, please use the scale 1–5 to indicate the level to which you agree with the following statements: 5, strongly agree; 4, agree; 3, neutral; 2, disagree; 1, strongly disagree. Relative to other more traditional science laboratory courses, I believe...

	<i>F'</i> 07 (<i>N</i> = 33)	<i>F'</i> 08 (<i>N</i> = 25)	<i>F'</i> 09 (<i>N</i> = 30)
5. The inquiry-based approach used in lab helped me better understand genetics concepts and methods (<i>e.g.</i> , mutation, gel electrophoresis).	4.27 ± 0.67	4.12 ± 0.78	4.47 ± 0.57
6. The inquiry-based approach helped me to make connections between different concepts/experiments.	4.00 ± 0.94	4.00 ± 0.82	4.33 ± 0.84
7. The inquiry-based approach helped me realize that research is interdisciplinary (<i>e.g.</i> , a mutation at the level of DNA affects the cellular/biochemical level; methods are required from more than one subject area— <i>e.g.</i> , genetics and chemistry).	4.27 ± 0.80	4.16 ± 0.69	4.37 ± 0.67
8. The concepts and technical skills I gained while participating in lab helped me/will help me in my further studies at Trinity and beyond.	3.73 ± 1.18	4.24 ± 0.72	4.10 ± 0.80
9. The concepts and skills I gained while participating in lab will help me in my <i>future career</i> .	3.58 ± 1.09	3.84 ± 1.11	4.07 ± 1.11

Questions Q5–Q7 queried students regarding course value relative to traditional labs in terms of understanding technical concepts and methods and the interdisciplinary linkage of scientific inquiry. Students generally agreed the approach was helpful (Q5, $\mu \geq 4.1$; Q6, $\mu \geq 4$; both over all 3 years). Results also suggest the labs helped students to see the multidisciplinary nature of scientific inquiry (Q7, $\mu \geq 4.1$ over all 3 years). Questions Q8 and Q9 assessed the value of the labs *vis-à-vis* students' futures at the institution and beyond. Most students follow a health professions track; here students felt the labs were helpful in achieving academic (Q8, $\mu \geq 3.7$ over all 3 years) as well as professional goals (Q9, $\mu \geq 3.6$ over all 3 years).

Year-to-year variation in survey results was observed, likely stemming from improved delivery of course material and participation by multiple faculty, although the labs were taught by a single instructor during year 3; notably all year 3 survey results had $\mu \geq 4$.

We believe the labs may be valuable in reaching goals that active, engaging teaching methods seek to achieve. We observe engaged students using the scientific pro-

cess in a real experimental context while simultaneously increasing their understanding of basic genetics techniques and concepts. Also, students see this lab as a valuable experience, and significantly, we find many of them having fun in the process.

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GENETICS

Supporting Information

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Using Student-Generated UV-Induced *Escherichia coli* Mutants in a Directed Inquiry Undergraduate Genetics Laboratory

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FILE S1**UV Mutagenesis of *Escherichia coli* and Analysis of Mutants****Equipment and Reagents**

Escherichia coli K12 bacterial strains

W3110 F, λ , IN(*rrnD-rrnE*)1
 W3110-*uvrA-phr* W3110 *uvrA277::Tn10 Δ phr-758::Km*

Plasmids

pBR322::*lacIZY* (Amp^r)
 pBR322::*lacZ* (Amp^r)
 pBR322::*lacY* (Amp^r)
 pET-15b (*lacI*, Amp^r)

Petri Dishes (35 mm for irradiation; 85 mm for routine plating and streaking of cultures)

Bacteriological Media

LB medium per liter

NaCl	5g
Yeast Extract	5g
Tryptone	10g
Agar (for plates)	15g

MacConkey Agar Medium (available from e.g., Fisher Scientific)

Antibiotics

Ampicillin Sodium Salt (available from Fisher Scientific or Sigma Aldrich)

Made up in water as 1000X 100 mg/mL concentrated solution store at -20C and
 use in medium at 100 μ g/mL

Kanamycin Sulfate (available from Fisher Scientific or Sigma Aldrich Chemical)

Made up in water as 1000X 50 mg/mL solution; use at 50 μ g/mL

ONPG (LacZ substrate; available from Fisher Scientific or Sigma Aldrich Chemical)

Light box (white light; optional; for identifying Lac⁻ mutants on MacConkey medium)

Chloroform

β -galactosidase (optional)

Z-Buffer (for β -galactosidase assays) per liter:

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 16.1 g

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 5.5g

KCl 0.75g

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.246g

β -mercaptoethanol 2.7 mL

Magnesium sulfate (for cell suspensions for irradiation; Fisher or Sigma Aldrich Chemical)

Micropipettes (P20, P200, P1000) and tips

Microfuge tubes and Microfuge

High speed centrifuge (for pelleting cells for irradiation)

37C incubator

37C shaker or rotator in 37C incubator

UV germicidal lamp, handheld UV lightsource or UV crosslinker

Plasmid Extraction Reagents (optional; commercial kits are also available from, e.g., Qiagen)

Alkaline lysis solution I

50 mM Glucose

25 mM Tris (pH 8.0)

10 mM EDTA (pH 8.0)

Alkaline lysis solution II

0.2 N NaOH (freshly diluted from a 10N stock)

1 % (w/v) SDS

Alkaline lysis solution III

60.0 mL 5 M potassium acetate

11.5 mL glacial acetic acid

28.5 mL H_2O

TE buffer (for storage of plasmid solutions)

10 mM Tris

1 mM EDTA

Agarose (available from e.g., Fisher Scientific)

Horizontal electrophoresis tanks and power supplies

50X electrophoresis buffer (50X TAE, use as 1X in agarose gels and running buffer) per liter:

Tris base 242g

Glacial acetic acid 57.1 mL

0.5 M EDTA 100 mL

(0.5 M EDTA is made up by dissolving 186.1 g disodium ethylene diamine tetraacetate in 800 mL water and adding ~20 g NaOH pellets to reach pH 8.0; make up to 1 liter final volume)

1X TSS Buffer (for plasmid transformation, from Chung et. al. 1989 PNAS 86:2172-2175):

LB medium containing:

10% w/v polyethylene glycol (mw 3350 or 8000)

5% v/v DMSO

50 mM MgSO₄ or MgCl₂

Thermal cycler

Thin-walled PCR tubes

PCR reagents (we use Qiagen Taq PCR MasterMix, a 2X concentrate to which we add primer,

DNA and water to make up to 50 µL reaction volume)

PCR primers

OLIGO	sequence, 5'–3'
lacI–f1	GAGTCAATTCAGGGTGGTGAA
lacI–r1	CGCTCACAATCCACACAAC
lacI–r2	ATACGAGCCGGAAGCATAAA
lacZ–f1	TGTGGAATTGTGAGCGGATA
lacZ–r1	TTTCCTTACGCGAAATACGG
lacZ–f2	CTTTATGCTTCCGGCTCGTA
lacZ–r2	TGTAGCCAAATCGGGAAAAA
lacZ–f3	GTGCGGATTGAAAATGGTCT
lacZ–f4	GGATGTCGCTCCACAAGGTA
lacZ–r3	CAGCAGCAGACCATTTTCAA
lacZ–r4	GACCTGACCATGCAGAGGAT
lacZ–r5	GGAGCGTCACACTGAGGTTT
lacZ–r6	GGGAAGTAGGCTCCCATGAT
lacY–f1	CCAGTTGGTCTGGTGTCAA
lacY–r1	TCAATGCGATCACTCCGTTA
lacY–f2	CATGTCTGCCCGTATTTTCG
lacY–r2	GCATGTTCAATGCGATCACT

Construction and growth of bacterial strains: *Escherichia coli* strains were cultured at 37° in Luria Bertani broth, or on Luria Bertani or MacConkey agar medium with added kanamycin (50 µg/mL), tetracycline (15 µg/mL), or ampicillin/carbenicillin (100 µg/mL) where appropriate. UV mutagenesis experiments were done using *E. coli* K12 strains W3110 and 3110-*uvrA-phr*. Strain W3110 is a wild type prototroph (F⁻, λ⁻, IN(*rrnD-rrnE*)1). The isogenic derivative strain W3110-*uvrA-phr* carries insertions in both the genes encoding components of the excision repair nuclease *uvrA* and in the DNA photolyase-dependent photoreactivation system *phr*. Strain W3110-*uvrA-phr* was constructed by using P1 transducing bacteriophage grown on appropriate host strains using standard transduction methods. P1 was first grown on strain JW0698 (Δ (*araD-araB*)567 Δ *lacZ*4787(::*rrnB*-3) Δ *phr*-758::Km λ⁻ *rph*-1 Δ (*rhaD-rhaB*)568 *hsdR*514), and bacteriophage lysates were used to transduce strain W3110 to kanamycin resistance to generate strain 3110-*phr*. P1 phage was then grown on strain N3055 (λ⁻ IN(*rrnD-rrnE*)1 *uvrA*277::Tn10) and lysates were used to transduce strain 3110-*phrA* to tetracycline resistance. One of the resulting transductants, designated W3110-*uvrA-phr*, was selected for the subsequent experiments.

Construction of plasmids for complementation analysis: Plasmid pLC20-30 from the Clarke and Carbon plasmid collection was digested with restriction endonuclease AclI and a 5.7 kb fragment carrying the *lacIZY* region was blunt-end using T4 DNA polymerase (New England Biolabs). Plasmid pBR322 was digested with EcoRV and ligated with the *lacIZY* fragment such that *lac* genes are expressed from the pBR322 *tet* promoter, yielding plasmid pBR322::*lacIZY*. Ligation products were used to transform NEBα competent cells (New England Biolabs). Transformants were selected on MacConkey agar with ampicillin to confirm Lac⁻ complementation.

To produce a *lacY* complementing plasmid, *lacY* was excised from pBR322::*lacIZY* by digestion with PvuII and BamHI. This fragment was ligated to pBR322 which had been digested with EcoRV and BamHI, resulting in plasmid pBR322::*lacY*. Ligation products were used to transform *E. coli* strain χ508 (λ⁻ *lacY*83 *glnV*42 T^R) and transformants were selected on MacConkey agar with ampicillin.

To produce a *lacZ* complementing plasmid, PCR primers *lacZF* (5'-aagcttacacaggaaacagctatga) and *lacZR* (5'-ggatccttattttgacaccagacca) were used to amplify the promoterless *lacZ* gene from W3110 genomic DNA. The resulting amplification product was digested with HindIII and BamHI and ligated into HindIII- and BamHI-digested plasmid pBR322. Ligation products were used to transform NEBα; transformants were selected on Luria Bertani agar medium with ampicillin. Plasmids were extracted from transformants and digested with HindIII. The pool of linearized plasmids was ligated to HindIII-digested *Zymomonas mobilis* genomic DNA, and ligation products were used to transform strain JW0698. Transformants were selected on MacConkey agar with ampicillin. Plasmids were extracted from transformants expressing strong Lac⁺ activity on MacConkey agar media; these plasmids, designated

pBR322::lacZ1, pBR322::lacZ2, etc., were used for complementation of *lacZ* mutations.

Complementation of *lacI* mutations is done using plasmid vector pET15b (Novagen) with selection on MacConkey agar containing ampicillin.

Week 1: Handling of bacterial cultures and dilution plating

Objectives and Overview

In this lab we will be preparing for your investigative experiment by practicing pipetting, aseptic, and bacterial culture techniques. These skills will be crucial to your success throughout the semester. Specifically, we will be learning how to do dilution plating of liquid cultures, including plate spreading, to get accurate counts of bacterial cell densities, how to streak from colonies for isolates, and how to inoculate tubes aseptically.

Background I, Dilutions and Dilution Plating

At times, the concentration of a solution may be too high for accurate measurement. If this is the case, accurate dilutions of the solution can bring the concentration into the necessary range, and the initial concentration can then be calculated by measurement of the diluted sample multiplied by the dilution factor.

To dilute a sample, we take a known volume of the sample and add it to a known volume of diluent (also known as a blank) that contains none of the chemical or component of interest. The dilution of the resulting sample is usually expressed as a fraction, and usually in whole amounts such as 1/10 (spoken as “one to ten”), 1/100, etc. to make later calculations easier. To calculate the dilution, the amount of the sample is put in the numerator and the total volume (sample + diluent) is in the denominator. For example, if we did add 1 ml of sample to 9 ml of diluent, the dilution is 1/10. The dilution factor (D) is the reciprocal of the dilution. In our example above, the dilution factor is 10.

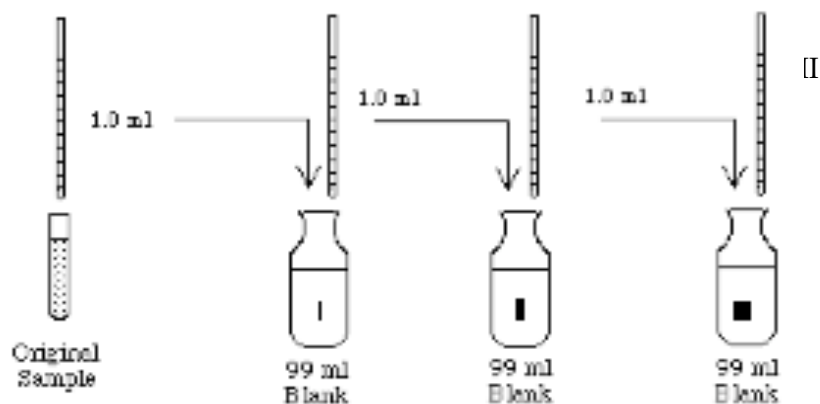
If we do not know the original concentration in a given sample, it is not practical to do one dilution and hope that this concentration is within the acceptable range, nor is it practical to do large-scale dilutions in one step (e.g. 1 M diluted to 1 μ M). In these conditions, we must dilute the concentrated solution several times in what is known as a serial dilution to get the dilution(s) needed. Hopefully, one of these should result in a measurable dilution, which can be used to calculate the amount in the original sample by multiplying the measurement of the dilution times its dilution factor. A serial dilution is illustrated on the following page.

There are some simple guidelines that you should follow when performing a serial dilution:

1. Label everything! It is very easy to confuse which container is which.

2. Mix well! You can reduce your experimental error by mixing each solution or dilution well before removing a portion for the next dilution. This is especially true with cells that settle to the bottom of the tube.
3. Be careful with your pipetting. Make sure quantities are accurately and consistently transferred from container to container.

Figure 1. Serial Dilution Scheme



Dilution	1/100	1/100	1/100
Dilution Factor	D=100	D=1×10 ⁴	D=1×10 ⁶

Each transfer results in a 1/100 dilution, so the sample in Bottle I has been diluted 100 fold. Bottle II is a 1/100 dilution of Bottle I, and these dilutions are multiplied to get the total dilution. If the original sample contained 8×10^8 cells/ml, Bottle I contains 8×10^6 cells/ml, Bottle II contains 8×10^4 cells/ml, and Bottle III contains 8×10^2 cells/ml. Any quantitative data obtained from a dilution, for example Bottle III, must be multiplied by the dilution factor to determine the concentration in the original sample, and you can see that calculating the original concentration using the reading from Bottle III gives the following: $8 \times 10^2 \times 1 \times 10^6 = 8 \times 10^8$

Background II, Aseptic technique and manipulation of microbes

One of the keys to being successful in the lab when working with microorganisms is to avoid contamination. This will be important for your experiments, so we will be practicing transferring bacteria to plates and liquid broths.

General Procedures

During lab you will make dilutions of a bacterial culture plate these dilutions onto agar media. In addition, you will be streaking plates and inoculating broths.

Protocol I: dilution plating of bacteria

For this protocol, you should work in your lab pairs making one set of dilution plates.

1. Make 10 mls of a 1:1000 dilution of the bacterial culture in LB-Kan.
2. Make 10 mls of a 1:100 dilution of your first dilution (what is the dilution factor for this tube?)
3. Plate 100 μ l of the dilution made in (2) onto an LB-Kan plate labeled with your names and 'A'. Note — it is easiest to observe what happens on a plate if you label the plate around the edge of the agar side.
4. Make a 1:10 dilution of the dilution made in (2) and then plate 100 μ l of this new dilution onto an LB-Kan plate labeled with your names and 'B'.
5. Make a 1:10 dilution of the dilution made in (4) and then plate 100 μ l of this new dilution onto an LB-Kan plate labeled with your names and 'C'.
6. Make a 1:10 dilution of the dilution made in (5) and then plate 100 μ l of this new dilution onto an LB-Kan plate labeled with your names and 'D'.
7. Make a 1:10 dilution of the dilution made in (6) and then plate 100 μ l of this new dilution onto an LB-Kan plate labeled with your names and 'E'.
8. Each team member should come back after 24-48 hours and make a count of all the colonies on a plate with a countable density. Use these data to calculate an estimate of the cells/ml in the original tube, and send your count data to your instructor.
9. After counting the cells on your plate, be sure to dispose of the plates in the biohazard box.
(your instructor will be looking at your plate series to grade for technique).

Protocol II: tube inoculation

Each person should complete this protocol independently.

1. Select a colony from one of the plates provided and use it to inoculate an LB-Kan tube. Write your name on the tube and 'inoculated'. Place the tube in the rotating incubator.
2. Mock inoculate a tube by going through the same procedure (flaming your loop, inserting it into broth, etc.), except that in this case you do not select a colony from the plate (this checks your aseptic technique). Write your

name on the tube and 'mock'. Place the tube in the rotating incubator (use this tube to balance your other tube).

Your instructor will be looking at your tubes to grade for correct technique.

Protocol III: streaking for isolates

Each person should complete this protocol independently.

1. Using aseptic technique, get a loopful of bacteria from the culture of Kan^r bacteria provided and streak out for isolates on a LB-Kan plate. Label the plate with your name and a code that will allow you to identify your plate and place it in the 37C incubator. Next do the same procedure with the Kan^r culture and an LB-Carb plate. Next do the same procedure with the Kan^r culture and a plate of LB agar with no added antibiotics. Finally, do the same procedures with the Kan^s culture and an LB-Kan, LB-Carb, and an LB plate. Your instructor will grade your plates for correct technique.

Week 2 UV Mutagenesis of wild type *lac*⁺ *E. coli* strain W3110 and UV-sensitive strain W3110-*uvr-phrA6*

Objectives and Overview

In this lab we will be using UV light to mutagenize strains of *E. coli* that are able to metabolize lactose (*Lac*⁺) to look for mutants which cannot use lactose as a carbon source (*Lac*⁻). These mutant strains will be the basis of our subsequent labs as we try to characterize which genes are mutated in each strain.

Background: Uses of mutants in genetic studies**A. Forward and reverse genetics**

Analysis of mutants is the main tool used by a geneticists to understand how the information in DNA is translated into the phenotype. These analyses can be broadly grouped into two categories, forward genetics and reverse genetics. Forward genetic analyses are the older of the two methods, but they are still a standard way of determining the genetic basis of a trait. In brief, the procedure for analyzing a trait using forward genetics is to find or create useful mutants, determine the number of loci affecting the trait by grouping mutants through crosses, then characterize representative mutants for each locus at as detailed a resolution as possible, in most cases down to the level of the DNA sequence.

The increasing availability of genome sequences and precise mutagenesis techniques in some organisms, even down to the level of knocking out genes in specific tissues or cells, has led to the development of reverse genetics. Reverse genetic analyses determine the function of a specific gene by knocking it out and observing the mutant phenotype. Once the gene(s) responsible for a trait has been identified, genetic studies can branch off in many directions by analyzing the biochemical functions of encoded proteins, determining the differences in tissue and/or temporal expression patterns, or assessing the amount of natural variation within or between species, etc.

In lab this semester we will be conducting a forward genetic screen to analyze the phenotype of lactose metabolism in *E. coli*. The *lac* operon is one of the most intensively studied genetic systems in the world. We will be spending a few days when we study prokaryotic gene regulation going over how the *lac* operon works, but in preparation for this lab it would be helpful to look at the figure illustrating the *lac* operon in your genetics textbook. Our goal is to be able to show what gene(s) were mutated in our strains, determine the sequences of the mutant loci, and propose hypotheses for why these mutations lead to a *Lac*⁻ phenotype.

B. Understanding your phenotype

When a geneticist embarks on a mutant hunt, one of the first things they need to do is understand the phenotype they are investigating. Thus far in class we have talked about phenotypes as being clearly distinct, but there may be substantial variation in a phenotype that is important to appreciate in order to define a mutant phenotype. For example, if

looking at Mendel's purple flower color, what if there was a lighter purple color? Would that be a mutant or maybe just due to more sun or fertilizer? As a geneticist, you need to be aware of what the wild-type phenotype is and what constitutes a meaningful deviation from that phenotype.

We are hoping to identify strains mutated in their ability to metabolize lactose. As you can imagine, it is hard to visualize the absence of something. One important part of the mutant hunt is to define assay conditions that allow for rapid phenotype screening and identification of mutant genotypes, which may be present at an extremely low frequency. We will be using MacConkey agar plates, which are described below, to visualize our phenotype.

Note: much of the following paragraph is taken from the American Society of Microbiology's Microbelibrary.org page.

One easy way to differentiate between different types of bacterial cells is based on their differential metabolic properties. For example, some cells may be able to utilize a particular carbon source, such as lactose, or produce a particular metabolic intermediate. The first example of a differential media was MacConkey agar which was developed by Alfred Theodore MacConkey, M.D. early in the 20th century. This medium selects for the growth of gram-negative microorganisms commonly found in the gut and inhibits the growth of gram-positive microorganisms, and also allows for the differentiation between lactose fermenting and non-fermenting bacteria. The bile salts in this media discourage the growth of gram-positive bacteria because the cytoplasmic membrane is sensitive to these salts; the tolerance of gram-negative enteric bacteria to bile is partly a result of the relatively bile-resistant outer membrane.

Gram-negative bacteria growing on the media are differentiated by their ability to ferment the sugar lactose as shown by a dye indicator. Bacteria that ferment lactose cause the pH to drop as acid end products are produced. This change in pH is detected by neutral red that the bacteria have absorbed from the media, which is red in color when $\text{pH} < 6.8$; if lactose is metabolized, the colonies will appear bright pink to red on the agar. The color of the medium surrounding a colony may also change. Strongly lactose fermenting bacteria produce sufficient acid to cause precipitation of the bile salts, resulting in a pink halo in the medium surrounding individual colonies or areas of confluent growth. Bacteria with weaker lactose fermentation growing on MacConkey agar will still appear pink to red but will not be surrounded by a pink halo in the surrounding medium. Gram-negative bacteria that grow on MacConkey agar but do not ferment lactose appear colorless on the medium and the agar surrounding the bacteria remains relatively transparent.

When looking for mutants it would be ideal to establish conditions such that your mutants are the only members of the population that are observable. A classic example of this is selecting for antibiotic resistance on growth medium

containing the antibiotic — only the resistant mutants will survive. This type of mutant identification is known as a selection. Unfortunately, we are looking for a loss-of-function mutation, and both the mutant and the wild type organisms are capable of growth, so we will need to rely on sharp eyes and a good understanding of the normal range of colony morphology to be able to pick out mutants based on colony coloration on MacConkey agar. This approach is known as a screen. We will provide you with a plate containing Lac⁺ and Lac⁻ colonies for your inspection, so you can see the differences between these genotypes on the media.

C. Mutagenesis methods

As stated above, geneticists are always on the lookout for interesting mutant phenotypes, but given the low frequency of most of these mutations in nature, we often need to resort to generating our own mutants in order to study a trait of interest. Your textbook outlines mutagenesis and you should see those sections to get an overview of mutagenesis prior to lab. We will be using UV light to cause random mutations in the *E. coli* genome. Cells naturally have the ability to repair damage to their DNA caused by UV damage, which reduces the efficiency of UV as a mutagen. As preparation for this lab, Dr. Healy has knocked out two of the genes necessary for repair, *uvrA* and *phr*, so that the cells we are using are repair deficient. We will also be treating the parent strain with UV, and part of the results of this lab will be a comparison of the DNA repair efficiencies of these two strains.

General Procedures

We will all be working together this week in lab to generate our mutants. After the first day, lab pairs will be working together to gather data on survival at the different time and distance combinations we used for mutagenesis, and then lab pairs will be working together to pick their putative mutants for further analysis. This lab will require some time outside of the normal period to check plates and count bacterial colonies and select mutants, so the lab will be open on subsequent days for you to come in. The anticipated time required outside of class will be approximately ½ hour per lab group on the first day after the mutagenesis and about 1 hour the second day after the mutagenesis. Below is a description of the activities for lab this week. It is not written in the form of a protocol, so it is up to you to write a detailed protocol of the activities you will undertake in lab.

Important safety tip: we will have a UV source on at all times during this lab. While you will be wearing safety glasses, please do not look at the lamp and limit your exposure to the lamp's rays!

Prior to lab your instructor will have grown the parental strain, called 3110, and the derivative double repair mutant strain (*uvrA⁻ phr⁻*) in overnight cultures. These cells will have been spun down and resuspended in 0.1 M MgSO₄ for the mutagenesis. Because we do not know *a priori* what the optimum set of parameters are for mutagenesis, we will be exposing the cells to UV for four different time points (10 seconds, 30 seconds, 60 seconds, and 120 seconds) at each of four distances from the UV source (10 inches, 25 inches, 45 inches, and 70 inches). We will be using 1.5 mls of cells in 35 mm culture dishes for each exposure. When labeling the culture dishes, please use the system described below that

enables you to see what the culture, distance, and time combinations are for each dish (note — you might want to label the top and bottom of the dish in case they get separated):

Strain	Distance	Time
1 = 3110	A = 10 inches	1 = 10 seconds
2 = <i>uvrA⁻ phr⁻</i>	B = 25 inches	2 = 30 seconds
	C = 45 inches	3 = 60 seconds
	D = 70 inches	4 = 120 seconds

Using this system, dish 1A1 is 3110 at 10 inches with a 10 second exposure and dish 2C3 is the *UvrA⁻ Phr⁻* repair mutant at 45 inches and 60 second exposure.

After the UV lamp is set to the correct height, gently swirl the dishes so that the cells are not all settled at the bottom. The set of eight dishes for that height should then be uncovered and placed under the lamp and the timer started. At each time point, remove the appropriate dishes and cover them. Be sure to remove the dish lids.

Once the set of exposures for a height is complete, you will be creating a dilution series for each strain × distance × time combination to obtain a count of the number of viable cells in these cultures. Take 100 μ l of cells and add this to 900 μ l of media to create the 10^{-1} dilution. Mix this tube and then take 100 μ l of this dilution and add it to 900 μ l of media to create the 10^{-2} dilution. Repeat this process until you have created the dilutions you need to plate out for each strain × distance × time combination shown in the table below:

W3110

10 s	10^3-10^6	10^3-10^6	10^3-10^6	10^4-10^7
30 s	10^3-10^6	10^3-10^6	10^3-10^6	10^4-10^7
60 s	10^2-10^5	10^3-10^6	10^3-10^6	10^3-10^6
120 s	10^1-10^4	10^2-10^5	10^2-10^5	10^3-10^6
	10"	25"	45"	75"

untreated, 10^5-10^8

uvrA⁻ phr⁻

10 s	10^1-10^4	10^1-10^4	10^1-10^4	10^2-10^5
30 s	10^0-10^3	10^0-10^3	10^0-10^3	10^0-10^3
60 s	10^0-10^3	10^0-10^3	10^0-10^3	10^0-10^3
120 s	10^0-10^3	10^0-10^3	10^0-10^3	10^0-10^3
	10"	25"	45"	75"

untreated 10^3-10^7

For each plating, spread 100 μ l of cells onto the surface of the plate as you have practiced in lab.

Each lab pair will be responsible to return after 24 hours to count colonies on your countable plates to get an estimate of the density of viable cells in the original cultures and treatments. To be able to see the colonies on the plates, hold the plate over a light source (a light box will be provided). To count the colonies, divide the plate in half and use a sharpie to 'dot' each colony that you count on the half of the plate. When you are done, use a little bit of ethanol to wipe off your marks (but not the plate name!) so that the next group can count colonies. Send your estimates of the original densities from each strain \times distance \times time combination to your instructor and we will then combine these estimates and return the data to you.

You will be using these data to create graphs showing the effect of exposure on cell viability. For each plate where we could get a count, find the ratio of the number of cells/ml in the original dish exposed over the number of cells/ml in the culture not subjected to UV. Plot these points on a graph with %survival on the Y axis and exposure time on the X axis. Use different lines for the different heights and cell genotypes. Create another graph showing the total estimated cell counts for each of these time points with cell number/ml on the Y axis and time on the X axis. Use different lines again for the different heights and genotypes and be sure to include error bars for the estimated cell counts.

The other important thing we will be doing is plating out cultures to look for mutants. To allow your mutagenized cells a chance to recover, you will be removing 0.2 mls of cells from the dish and adding this to a tube containing 0.8 mls of LB broth, and then incubating the bacteria at 37°C for 1 hour. Based on experience from previous years, we will be doing this for the 2B1, 2B2, 2C1, 2D1, and 2D2 tubes, and it would be best to make as many of these subcultures as possible to give us the greatest chances of finding mutants. Use the same designations as above, but add -1, etc. to indicate this is the first dilution (e.g., 2B1-1). After one hour, plate out as many of the following on MacConkey-Kan agar as you can:

2B1	100 μ l of a 1:10 dilution
	100 μ l of a 1:100 dilution
2B2	100 μ l of a 1:10 dilution
2C1	100 μ l straight from the culture tube
2D1	100 μ l of a 1:100 dilution
2D2	100 μ l straight from the culture tube

When plating, please record which tube you are using as a source, and then add another number for indexing (e.g., 2B1-1-1 is the first plate, 2B1-1-2 is the second plate, etc). To plate out the cells add the liquid to the plate and then use a sterilized cell spreader (dip in 100% ethanol and then pass over a flame) to push the liquid evenly over the surface of the plate.

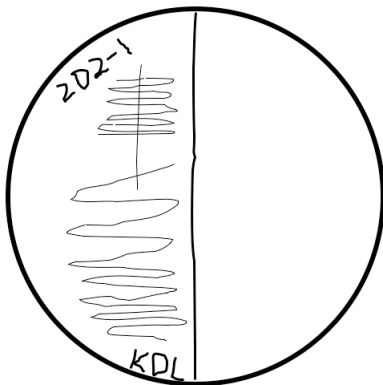
Incubate the plates overnight at 37°C.

After an overnight incubation, these plates will be removed from the incubator. It will be up to you and your lab partner to look for mutants. When scanning your plates for mutants, try to open the lids as little as possible. These plates contain kanamycin to select against contaminants, but it is good practice to take measures to minimize contamination. If the lid of your plate has condensation on it, you can use a clean kimwipe to remove the condensation so that it is easier to scan the plate. Each lab group should try to pick eight mutant colonies.

As you look at these plates, first take a global view. Do they all look the same? If so, the mutation frequency might be too low to find mutants of interest. Variation in colony morphology is a good sign that many of the founder bacteria carried mutations in their genes. As you start to look more closely at the bacteria, try to define for yourself significant aspects of the colony phenotype so that you can recognize a mutation of interest against the background “noise.” Things to consider are colony color, colony shape, color of the media around the colony, etc. When you identify a mutant, turn the plate over and circle it.

With all of your mutants identified, you will next want to transfer them to a new plate. We subculture the organisms to make sure that we have a pure culture, i.e. that we don’t have a mixture if a mutant colony is touching another colony or a similar situation. You should at this point give each mutant a unique identifier. In our case, the mutants will be coming off of a series of plates that originated from different treatments — so you will want to incorporate that into your identifier. For example, if you have a series of mutants found on plates from the 2D2-1-1 treatment, you could name them 2D2-1-1a, 2D2-1-1b, 2D2-1-1c, etc.

Next you will want to prepare your plates for subculturing. It is best to use agar plates at room temperature, so if you are taking a plate out of the refrigerator you should allow it time to warm to room temperature. You do not need to use an entire plate for subculturing — you can split a plate in two or more sections as diagrammed below. You should put your name on each plate and the name of the organism you are subculturing. Remember that it is easiest if you label your plates around the edges of the plate. The diagram below is a reminder of how to streak out for isolated colonies on a plate:



1. Label and divide your plate.
2. Flame your loop, allow to cool, then pick colony off of source plate and do zig-zag streak seen at the top.
3. Flame your loop, allow to cool, then do vertical streak down through the zig-zag streak.
4. Flame your loop, allow to cool, then cross the vertical streak once or twice and do another series of zig-zag streaks.

You can repeat this twice on the plate. What this should do is give you isolated colonies along your last streak. If there is more than one bacterial clone in the original colony you selected, you should see two different colony types in the area where you have single colonies.

You should then put all of your plates in the 37°C incubator for an overnight incubation. When you check on the plates, make notes of what you see. The source plates you used should be left out on the benchtop until you are sure that your colonies have grown.

When you check the plates after their overnight growth, you should hopefully see isolated colonies that will allow you to determine whether you have true mutants! For each true mutant you have identified, you will want to repeat the procedure above to get a plate where you have a pure culture of each mutant on half of the plate.

You will then want to set up a system for keeping each of the mutant strains that you have, which will involve restreaking them out every four days to maintain their viability. What you should do is come in every four days and take a loopful of inoculum from several colonies and transfer to a new plate, incubate that plate overnight to make sure they grow, then keep that plate on the benchtop for three days until you subculture it again. In this case we take a loopful of bacteria from several colonies to make sure we don't happen to pick a colony that has an additional mutation or something else that might confound our experiments. Be sure to not throw away any of your old plates until you are sure the new culture is growing and pure!

Week 3 β -galactosidase assays of mutant cultures

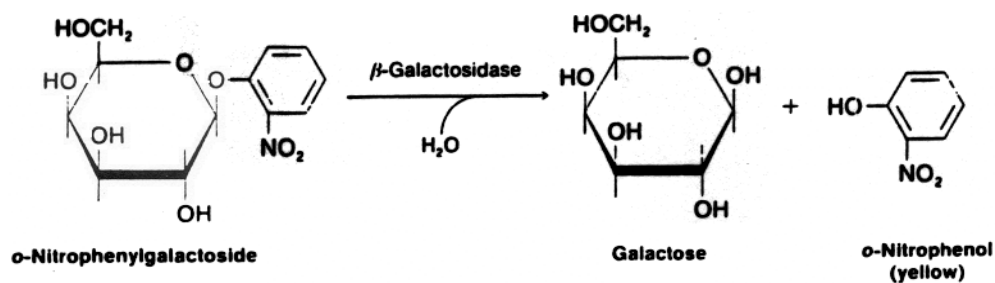
Objectives and Overview

In this lab we will begin narrowing down the causal basis of our mutant phenotype by testing our mutants for β -galactosidase activity. We will also be testing for inducibility of our mutants by incubating them in the presence and absence of IPTG, a chemical analog of lactose that cannot be metabolized by the cell, but which does depress expression of *lac* genes

Background

After initial identification of a mutant, a geneticist usually will try to use additional, more sensitive assays to refine the phenotypic definition of each mutant. For example, our mutants were selected on the basis of a visible colony phenotype. Using a colony phenotype allowed us to scan many bacterial clones, but it is a crude assay in that there might be many reasons for a colony to be Lac⁺. The *lac* operon has been the subject of intense study for over fifty years (see your textbook for an overview of the *lac* operon). We can use the results of these previous studies to try to narrow down the causes of our mutants' Lac⁺ phenotype. In today's lab we will be testing explicitly for both LacZ and LacY activity. The *lacZ* gene encodes β -galactosidase, the enzyme that catalyzes the hydrolysis of lactose into glucose and galactose. The cleavage of lactose is a reaction that can be measured, but the standard assay to differentiate these sugars is rather insensitive and laborious. We can, however, use a synthetic substrate, *o*-nitrophenyl- β -D-galactoside (ONPG) to assay for LacZ activity. ONPG is colorless, but upon hydrolysis by β -galactosidase the molecule is cleaved into galactose and *o*-nitrophenol (see figure below), and the *o*-nitrophenol compound is yellow, allowing us a means to visualize LacZ activity.

The *lacY* gene encodes an integral membrane protein, known as a permease, which is a transporter used to bring lactose into the cell. We will be measuring LacY activity indirectly, by looking at the LacZ activity of intact cells versus cells which have had their membranes permeabilized with chloroform.



Another hallmark of the *lac* operon is inducibility. The genes of the operon are not normally expressed unless lactose is present in the environment. We can test for inducibility of our mutants by growing them in the presence and absence of lactose and assaying for gene activity. An alternative to lactose as an inducer is isopropyl- β -D-thiogalactoside (IPTG), which can be advantageous in experiments because it cannot be metabolized by the cell and so leads to continuous expression of the *lac* genes. In this lab we will be testing for inducibility of our mutants by growing them in the presence and absence of IPTG and then assaying for LacZ and LacY activity as described above.

YOU MUST COME IN 24 HOURS PRIOR TO LAB TO INOCULATE YOUR CULTURES, OTHERWISE YOU WILL NOT HAVE ANYTHING TO DO DURING LAB AND WILL LOSE PERFORMANCE POINTS!

General Procedures:

Your mutant cultures, along with positive and two negative controls (both *lacZ*⁻ and *lacY*⁻), will need to be grown for 24 hrs prior to lab, and then we will be using these cultures to inoculate media with and without IPTG for 3 hours of growth. These cultures will be pelleted by centrifugation and resuspended in LacZ assay buffer (known as Z buffer) and then tested for gene activity by addition of ONPG, chloroform, and exogenous β -galactosidase.

Protocol:

Note that you will be treating many tubes in parallel. One important key to this lab will be to establish a system of numbering your tubes so that you can keep track of what is in each tube quickly and easily.

- 2. 24 hours prior to lab, come in and inoculate a snap cap tube of LB-Kan with your mutant and carefully inoculate tubes of LB with colonies from the wt (3110 strain) and two Lac mutant strains (one is *lacZ*⁻ *lacY*⁺ and the other is *lacZ*⁺ *lacY*⁻). These plates will be in your lab's area on the far right-hand benchtop (as you stand with your back to the chalkboard). Please be careful to not contaminate these plates as they do not have antibiotics on them. This means you should also be careful in inoculating your tubes as they do not have antibiotics in them either. Be sure to click the caps of the tubes shut, and you should have a total of four tubes in the rotator in the incubator.
- 1. 3 hours prior to lab, use 175 μ l from each of your overnight cultures to inoculate both an LB and an LB-IPTG tube. Again, be sure to exercise aseptic technique and click the lids of the tubes shut. You should now have eight tubes in the rotator in the incubator.
1. In lab, transfer 0.5 ml cells from each tube into each of six 1.5 ml microfuge tube and spin cells at 8000 rpm for 5 minutes (you should have 48 small tubes at this point, and the remaining instructions will be for what you should be doing for each of these tubes). If you do not see a cell pellet, be sure to spin the cells again.
2. For each 1.5 ml tube, carefully pour off supernatants into waste container and then pipette off any remaining supernatants.
3. Add 1.0 ml Z buffer to each tube and vortex cell pellets until they are broken apart.

4. Spin cells again at 8000 rpm for 5 minutes, remove supernatants, and resuspend each pellet in 1.0 mls of Z buffer by vortexing.
5. To start your treatments, add CHCl_3 , β -galactosidase, and then ONPG to the tubes as indicated in the table below. Keep the cells at room temperature for 20 minutes with periodic swirling of the tubes. Note that the color changes may start to occur while you are adding solutions to other tubes, so refer back to your tubes often while you are pipetting. It may help you to see the changes if you place your tubes on a sheet of white paper during the incubation.

	Tube					
	1	2	3	4	5	6
CHCl_3				X	X	X
β -gal		X			X	
ONPG	X	X		X	X	

Key:

CHCl_3 = add 1 drop of chloroform (this permeabilizes the cell membrane)

β -gal = add 1/40 volume of 100 $\mu\text{g}/\text{ml}$ β -galactosidase in Z buffer

ONPG = add 1/10 volume of 10 mM ONPG in Z buffer

6. Observe the color change or lack thereof in each of the treatments, including how quickly the change occurred, and interpret whether there is LacZ and/or LacY activity and whether your mutants are inducible. Note, the *lacY*⁻ phenotype is most visible when comparing tubes 1 and 4 from the uninduced samples. The questions below may help you to analyze your results. If you wish, you can let your samples continue to incubate overnight on the benchtop to see what extra time will show.

Questions to help guide your analysis of this week's experiments:

1. What does tube 3 show? Is this a positive or negative control?
2. What does tube 6 show? Is this a positive or negative control?
3. What does the comparison of tubes 3 and 6 show? How is this a control?
4. What does tube 5 show? Is this a positive or negative control?
5. What does tube 2 show? Is this a positive or negative control?
6. What does the comparison of tubes 2 and 5 show? How is this a control?
7. What does tube 1 show?

8. What does tube 4 show?
9. What does the comparison of tubes 1 and 4 show?
10. What does the comparison between the induced and uninduced samples show?
11. Which tubes are important to observe in the comparison of the induced and uninduced samples?
12. Why is it important to do all of these comparisons for all three of the strains in addition to your mutants?

Week 4 Plasmid Isolation

Objectives and Overview

One way of showing that a gene is responsible for a particular phenotype is through complementation, where a wild-type copy of the gene is introduced into a mutant to restore the wild-type phenotype. In this lab you will be using a plasmid DNA isolation procedure to obtain three different plasmids, one with a *lacZ⁻ lacY⁺* genotype, another with a *lacZ⁺ lacY⁺* genotype, and a *lacZ⁻ lacY⁻* control. After isolating the plasmids, we will be checking our yield with a spectrophotometer. Next week you will be using your plasmid DNA to transform your mutant lines to show functional complementation of your *Lac⁻* genotype.

Background I, Plasmids and Molecular Biology

Plasmids are small, circular molecules of DNA that are used to move and copy specific pieces of DNA in recombinant DNA experiments. In order to be able to work with plasmids, scientists need to be able to get plasmids out of bacterial cells, modify them, and put them back in cells. Your textbook has good explanations of plasmids, cloning and transformation and you may wish to review these sections prior to lab to refresh your memory. Prior to this lab, Dr. Healy was able to take a clone containing the entire *lac* operon and move it to another plasmid containing ampicillin resistance, as well as make a *lacY⁺* subclone. These new clones will allow us to transform our bacteria and select for only those bacteria that took up the plasmids by including ampicillin in the media. By selecting for ampicillin resistance on MacConkey media, we will be able to tell if the plasmids complement our mutant genotypes.

Background II, Plasmid isolation

Although there are many different methods available for plasmid isolation, most have the following steps in common:

1. Growth and harvesting of plasmid-containing bacterial cells. The cells from an antibiotic-resistant colony are usually grown overnight in a liquid culture to stationary phase and then harvested as a pellet by centrifugation.
2. Lysis of the bacteria. Lysis (breaking open) of the bacteria is frequently brought about by agents that destroy the bacterial cell wall and plasma membrane.
3. Purification of the plasmid DNA. Isolating the plasmid DNA from the lysate often involves differential precipitation, where the bacterial components (chromosomal DNA, proteins, and membrane fragments) are removed by centrifugation, while the smaller plasmid DNA remains in solution.

4. Concentrating plasmid DNA. Nucleic acids including plasmid DNA can be precipitated by alcohol in the presence of salt (e.g. ethanol, isopropanol), and alcohol precipitation is frequently used to concentrate plasmid DNA following purification.

The technique we will use is a modification of the alkaline lysis method from Molecular Cloning, A Laboratory Manual, 3rd Ed. This technique utilizes the detergent sodium dodecyl sulfate as a lysis reagent and also NaOH to make the lysate highly basic. The alkaline conditions lead to rapid denaturation of the plasmid and chromosomal DNA. The alkaline lysis step is followed by a rapid neutralization which results in reannealing of the small, relatively simple, circular plasmid DNA but causes aggregation of the large, complex chromosomal DNA. The aggregates, which also include precipitated protein and other impurities, are removed by centrifugation and the remaining “cleared lysate” is composed of highly purified plasmid DNA and RNA. The plasmid DNA is then precipitated with EtOH.

Protocol I, Isolation of Plasmid DNA by Alkaline Lysis

YOU MUST COME IN THE AFTERNOON PRIOR TO THE LAB TO INOCULATE YOUR CULTURES SO THEY CAN GROW FOR APPROXIMATELY 16 HOURS.

1. Transfer 1.5 mL of bacterial culture to a 1.5 mL microfuge tube. Be sure to keep this and all other solutions and intermediates on ice unless specified otherwise, and be sure to label all your tubes!
2. Centrifuge the bacterial suspension at room temperature for 2 minute at maximum speed. You should see a small cell pellet in the bottom of your tube.
3. Carefully and quickly remove all the liquid and dispose of it in the liquid waste container. If your cell pellet is firm, you can pour off the majority of the liquid and then use a pipette to carefully remove the rest.
4. Mix the cell pellet vigorously by vortexing, then add 100 μ L of ice-cold Alkaline lysis solution I and vortex again.
5. Immediately add 200 μ L of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times, making sure the entire surface of the tube comes in contact with the solution. *Do not vortex!* Store the tube on ice.
6. Add 150 μ L of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III throughout the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3–5 minutes. You should see a white, flocculent precipitate after this step.
7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at room temperature in a microfuge. Transfer the supernatant to a new 1.5 ml tube.
8. Precipitate nucleic acids from the supernatant by adding an amount of room temperature 100% ethanol equal to 2X the volume of supernatant recovered in step 7 (this is often abbreviated “2 volumes of ethanol”), mixing by inversion a few times, and letting the solution sit at room temperature for 5 minutes.

9. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at room temperature. It helps to orient your tubes in the rotor with the hinge side up. This will help you to know where your pellet should be (it may be hard to see).
10. Gently and quickly remove the supernatant, being careful not to disturb the pellet.
11. Allow the pellet to dry by inverting the tube on a Kimwipe on your bench.
12. Add 200 μ l of TE with RNase to your sample to redissolve it and let sit for 15 minutes at room temperature.
13. Add 20 μ l of 10 M ammonium acetate and 400 μ l of 100% ethanol to your sample and let sit for 5 minutes at room temperature to precipitate the plasmid.
14. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at room temperature (remember the hinge!)
15. Gently and quickly remove the supernatant, being careful not to disturb the pellet.
16. Allow the pellet to dry by inverting the tube on a Kimwipe on your bench.
17. Add 1 mL of 70% ethanol and gently inverting the closed tube several times to wash the pellet.
18. Recover the nucleic acid by spinning at room temperature for 2 minutes at maximum speed.
19. Repeat steps 17 and 18 from above and allow to dry completely until no drops of liquid are seen in the tube.
20. Dissolve the nucleic acid pellet by adding 50 μ L of TE (pH 8.0) and pipetting gently up and down a few times. Keep your plasmid on ice from now on!
21. Your instructor will show you how to quantitate your nucleic acid yields using the nanodrop spectrophotometer. You should record these readings in your lab notebook. Be sure your tubes are labeled and then put them in the freezer for next week.

Recipes:

Alkaline lysis solution I

50 mM Glucose

25 mM Tris (pH 8.0)

10 mM EDTA (pH 8.0)

Alkaline lysis solution II

0.2 N NaOH (freshly diluted from a 10N stock)

1 % (w/v) SDS

Alkaline lysis solution III

60.0 mL 5 M potassium acetate

11.5 mL glacial acetic acid

28.5 mL H₂O

Week 5 Agarose gel electrophoresis, spectroscopy, and complementation of Lac- mutant strains

Objectives and Overview

One way to demonstrate that a bacterial strain is mutated in a particular gene is to introduce a wt copy of that gene into a mutant strain to see if the gene restores the wt phenotype. If a gene is able to restore the wt phenotype, it is said to complement the mutant. This week we will be using the plasmids isolated last week with different *lac* genes in them to test which genes, if any, can complement your mutants. We will also use agarose gel electrophoresis to observe plasmids.

Background, Gel electrophoresis

A common way to analyze DNA samples is gel electrophoresis. At neutral pH, nucleic acids have a net negative charge because of the negative charges on the phosphate-sugar backbone, and because of this charge they will migrate when subjected to an electric field. The principle behind gel electrophoresis is to set up a matrix with pore sizes large enough to allow the nucleic acids to pass through, place the nucleic acids on one side of this matrix, then subject the gel and samples to an electric field. Under these conditions, nucleic acids loaded in sample wells at the negative electrode end of the gel migrate within pores of the gel matrix towards the positive electrode, and larger molecules move more slowly through the gel than smaller molecules. The end result is that molecules can be sorted according to size.

Agarose gels are a common type of stabilizing medium/buffer matrix used for the electrophoretic separation of nucleic acids. Agarose is a natural polysaccharide of galactose and 3,6-anhydro-galactose derived from agar (purified from seaweed). An agarose gel is made by boiling powdered agarose in a buffer solution that provides the ions necessary to conduct the current, and then pouring the mix into a casting tray. Before the agarose can polymerize, a comb is inserted to create wells where the nucleic acid can be loaded, and the agarose/buffer is then left to cool to room temperature and solidify. The resolving power of agarose gel electrophoresis is dependent on the pore size of the gel, which in turn is dictated by the concentration of dissolved agarose. High percentage agarose gels (e.g., 2%) are used for the separation of small DNA molecules (10^2 – 10^3 base-pairs in length) or molecules with small size differences, while low percentage gels (e.g., 0.6%) are used for large molecules (10^4 – 10^5 base-pairs) and/or larger size differences.

To 'run' a gel, the agarose gel is submerged in the same type buffer used in making the gel inside the electrophoresis tank. The samples to be separated are then prepared so they can be loaded into the sample wells. In order to visualize what you are pipetting and make samples denser than the buffer so that they sink to the bottom of the well, samples are mixed with a loading dye that has bromophenol blue and glycerol, usually in a 1:2 – 1:3 ratio of dye to total sample volume (e.g., add 5 μ l of dye to 10 μ l of sample). The dye also enables the investigator to follow the progress of an electrophoretic run because they migrate to the anode with a known rate. Once the samples are loaded, current from the

power supply travels to the negative electrode (cathode), supplying electrons to the conductive buffer solution, gel and positive electrode (anode), completing the circuit and forcing the DNA to migrate through the gel matrix.

Nucleic acids are not visible to the naked eye, and therefore it is necessary to make them visible in some way in order to see them in the agarose gel after electrophoresis. The most common method involves staining the nucleic acids with ethidium bromide (EtBr), and then detecting the DNA or RNA-bound dye with ultraviolet lamps. Staining with EtBr involves the “intercalation” of planar EtBr molecules between stacked nitrogenous bases in the DNA helix. Because of this property, EtBr is a mutagen (and probably a carcinogen); in addition, UV light can cause serious eye and skin burns. Therefore, gloves must be worn when loading or handling gels containing EtBr to limit direct exposure to EtBr and UV-filtering safety glasses must always be worn around the UV light sources.

In order to infer how large the different DNA samples on a gel are, each gel also has a DNA size marker loaded on to it. The size marker contains pieces of DNA of known size, and by measuring the distance migrated by these pieces and an unknown, the size of the unknown can be calculated.

Note to instructors: Students can use spectrophotometry or Nanodrop-type instrument to determine the concentration of plasmids; also PCR products.

Background, Bacterial cell plasmid transformation

Transformation is the uptake of naked DNA and its subsequent stable maintenance in bacteria. Some bacteria can be naturally transformed with DNA, and these bacteria are said to be naturally “competent”. The most commonly used cloning host, *E. coli*, is not naturally transformable, however, therefore *E. coli* must be made artificially competent by treating the cells with Ca^{2+} or other divalent cations. When *E. coli* cells are suspended in calcium chloride solution, their membranes are altered such that the cells are more likely to take up plasmid DNA molecules. Again, only circular DNA molecules will be stably maintained in *E. coli*; linear DNA is rapidly degraded. The transformation procedure provides a means for preparing large amounts of specific plasmid DNA, since one transformed cell gives rise to a clonal population of cells that contains exact replicas of the parent plasmid DNA molecule. This procedure was used to generate the bacterial strains used last week. As you saw, following growth of the bacteria in the presence of the antibiotic or some other regime that *selects* for the presence of the plasmid, the plasmid DNA can readily be isolated from the bacterial culture.

Transformation can also be used to put functional copies of genes into mutant cells to see if these genes can restore wt function. If the plasmids have intact coding sequences with the proper promoters, these genes can be expressed inside the cells to produce their encoded proteins. In this way we can test which genes are mutated in a particular strain.

General Procedures:

We will be doing three different transformations, a plasmid with *lacZ*⁺ *lacY*⁺, *lacZ*⁻ *lacY*⁺, and a *lacZ*⁻ *lacY*⁻ control. You should think through what you would expect to see if your mutant is a *lacZ*⁻ mutant or a *lacY*⁻ mutant before coming to lab. We will also be running a gel to check on your plasmid yields from last week.

Protocol:

16 HOURS PRIOR TO LAB YOU WILL NEED TO COME IN AND INOCULATE ON LB-KAN TUBE FOR EACH OF YOUR MUTANTS SO THEY CAN GROW OVERNIGHT.

Approximately 3 hours prior to lab you will need to come in and subculture your mutants by transferring 100 μ l of your overnight culture(s) to two new 2 ml tubes of LB-Kan (a 5% inoculum). You need to do this so we can have log-phase growth cells at the start of lab.

In lab:

1. Plate out 100 μ l of cells on Mac-Amp as a negative control.
2. Transfer 1.0 mls of fresh cells to a microfuge tube (make 1 tube of cells for each transformation you will be doing) and spin 7K rpm for 5 minutes (=5') or longer until the cells are pelleted (you may need to spin for 10' at 8K rpm).
3. Remove the supernatant and resuspend cells in 100 μ l of ice cold 1X TSS buffer by flicking your tube gently.
4. Add 3 μ l of your plasmid solution to the cells and mix gently with the tip of your pipettor.
5. Let the cells incubate for 40' on ice (no mixing during the ice incubation).
6. Add 1 ml of room temperature LB.
7. Incubate cells in a shaking incubator for 1 hr. (During this time we will be running a check gel of our plasmids)
8. Plate out 100 μ l of cells on agar with appropriate antibiotic and grow O/N. Keep the remainder of your cells out on the benchtop O/N just in case!
9. Come in after 20 hours or so to check on your plates and interpret your results.

This lab is based on a protocol published in Chung, C. T., Niemela, S. L., and Miller, R. H. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci 86:2172-2175

Week 6 PCR of suspected mutant genes

Objectives and Overview

In this lab we will be using PCR primers designed to amplify the genes that you think are mutated in each of your Lac⁻ lines. We will be using these PCR products as templates for sequencing reactions to see whether we can detect the particular based that have been mutated in your strains. You should go over the evidence you have with your instructor to make your choices, and as a control, everyone will also be amplifying the *lacI* gene in their strain.

General Background I, Polymerase chain reaction

The polymerase chain reaction (PCR) is a revolutionary technique developed by Kary Mullis, for which he later shared the 1993 Nobel Prize in chemistry. PCR has evolved to be the workhorse technology of the DNA age, with an almost limitless range of applications from medical diagnostics, to forensics, to genetic engineering, to biosensor technology. PCR is conceptually very simple, based on the observation that DNA polymerases can initiate replication of a single stranded DNA template if provided with (i) a short synthetic complementary DNA/RNA primer annealed and to the template strand, (ii) deoxyribonucleoside triphosphates, and (iii) the appropriate enzyme and reaction conditions. On a late night drive, Mullis realized that if double stranded (ds) DNA was used as a template with oligonucleotide primers specific for each strand, oriented convergent to each other, the single template molecule would be converted to two new dsDNA molecules (see section 9.4 in your text). Subsequent use of these two new dsDNA molecules for the same reaction would yield four copies of the dsDNA, etc. This *in vitro* DNA synthesis, called amplification, leads to a geometric increase in the amount of the dsDNA molecule, allowing us to generate many copies of a DNA sequence of interest from very little source material. PCR is very specific in that only the sequence located between the two primers is amplified. In most cases, knowledge of the sequence of the DNA of interest is a prerequisite of PCR in order to design synthetic primers.

In practice, PCR is performed by placing a test tube with the needed reagents in a machine called a thermalcycler. This machine cycles this tube between (i) high heat (95°C) to denature the dsDNA, (ii) a lower temperatures (~ 55°C) to facilitate annealing of the primers to complementary target sequences, and (iii) the optimal temperature for DNA polymerase activity. Most DNA polymerases (as well as many other proteins) are inactivated at 95°C, so the first PCR protocols required fresh DNA polymerase at the beginning of each cycle. A subsequent improvement for PCR was the incorporation of heat-stable DNA polymerases. Thermophilic and extremely thermophilic prokaryotic organisms (thermophiles and hyperthermophiles, respectively) have growth temperature optima of ~ 80°C and ~100°C, and their polymerases are able to maintain activity throughout the temperature fluctuations in PCR. Thus, while originally each PCR required a group of overworked and underpaid students with stop watches, pipettors, and water baths, now programmable thermalcyclers and thermostable enzymes allow rapid, repeatable amplification of template DNA.

The chemical conditions for PCR can be varied, but include the following essential components; (i) a pair of appropriately designed synthetic oligonucleotide primers, (ii) ample deoxyribonucleoside triphosphates (dNTPs) as substrates for DNA synthesis, (iii) buffer (pH 7.0), (iv) MgCl_2 to provide the of Mg^{++} cofactor required for the DNA polymerase, (v) a salt such as KCl required for optimal enzyme activity (vi) a nonionic detergent (Triton X-100) to keep most proteins from aggregating, (vii) DNA template, and (viii) the thermostable polymerase (the most common form is isolated from the organism *Thermus aquaticus* and called Taq polymerase). PCR conditions often must be optimized by altering the concentration of nucleotides, primers, Mg^{2+} , or temperatures of the different steps in the cycle.

General Background II, Design of PCR primers

The advent of easy, low cost DNA sequencing has led to the ready availability of many millions of DNA sequences from hundreds of thousands of species. The availability of known sequences allows us to design PCR primers to amplify specific sequences of interest. In practice, this usually means going to a database, extracting the sequence of interest, then using a computer program to assist in the selection of PCR primers by scanning for primers that have approximately the same melting temperature, exhibit low self- or cross-complementarity, and flank the sequence of interest.

As an example, here is how the primers were designed for today's lab. While it is not necessary for you to go to the websites before lab to recreate the sequences, it is a useful exercise to see how this procedure is done.

I went to the Entrez site, browsed the genome projects to find microbial genome projects, then found the W3110 sequence:

<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=89106884>

On this page I searched for "lac" and found the

lacI (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=AP_000997.1)

lacZ (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=AP_000996.1)

and *lacY* (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=AP_000995.1) sequences

Note that these are described as "complements" to sequences in the CDS field — this means that the gene is coded for on the opposite strand that was sequenced! In order to find the sequence of the gene, we will need to reverse-complement the sequence we get from Genbank.

The sequences we need are as follows:

lacI 365652..366734

lacZ 362455..365529

lacY 361150..362403

The next step was to go to the Genbank record for the entire W3110 genome sequence (ID AP009048) and then use the “Display:FASTA” to get the entire DNA sequence (all 4.5 million bases!!!) Then, I used the “Send to:File” to save it on my computer.

Once I had the file, I had to use a text editor to extract out the approximate sequence I wanted. Based on counting characters and lines, I decided to cut out from bases 360,011 to 367,990 so that I would have a bit extra on either side. Once I had cut out these lines (based on 70 characters per line, lines 5144–5257 in the file), I used a website to make the reverse complement to this sequence:

http://bioinformatics.org/sms/rev_comp.html

and I saved that to a file. Next I made a drawing that showed where each of the points in the sequence was that I needed (the start and stop points for each of the genes, based on the new numbering, 1–7980, from the reverse complement). You should produce a similar figure in your notebook prior to lab showing the original numbering, new numbering, and start and stop base numbers of each gene.

Finally, I took the sequence and used a program called Primer3 on the web to design my primers (<http://frodo.wi.mit.edu/>). Other programs exist, but this one is free and has always worked well for me. In particular, many other people like to use the program NetPrimer

(<http://www.premierbiosoft.com/netprimer/index.html>)

Here is the modified output from Primer3:

OLIGO	start	len	tm	gc%	any	3'	seq, 5'-3'
lacI-f1	1235	21	59.96	47.62	4.00	2.00	GAGTCAATTCAGGGTGGTGAA
lacI-r1	2434	20	60.16	50.00	4.00	0.00	CGCTCACAATTCACACAAC
lacI-r2	2414	20	59.70	45.00	4.00	0.00	ATACGAGCCGGAAGCATAAA
lacZ-f1	2419	20	60.07	45.00	4.00	2.00	TGTGGAATTGTGAGCGGATA
lacZ-r1	5581	20	60.09	45.00	6.00	1.00	TTTCCTTACGCGAAATACGG
lacZ-f2	2394	20	60.36	50.00	4.00	2.00	CTTTATGCTTCCGGCTCGTA
lacZ-r2	5691	20	60.43	40.00	3.00	0.00	TGTAGCCAAATCGGGAAAAA
lacZ-f3	3467	20	59.94	45.00	3.00	0.00	GTGCGGATTGAAAATGGTCT
lacZ-f4	4474	20	61.07	55.00	4.00	2.00	GGATGTCGCTCCACAAGGTA
lacZ-r3	3493	20	59.99	45.00	3.00	3.00	CAGCAGCAGACCATTTTCAA
lacZ-r4	3562	20	60.08	55.00	4.00	2.00	GACCTGACCATGCAGAGGAT
lacZ-r5	4655	20	60.31	55.00	5.00	1.00	GGAGCGTCACACTGAGGTTT

lacZ-r6	5670	20	60.29	55.00	4.00	3.00	GGGAAGTAGGCTCCCATGAT
lacY-f1	5512	20	59.57	50.00	4.00	1.00	CCAGTTGGTCTGGTGTCAAA
lacY-r1	6910	20	60.22	45.00	4.00	2.00	TCAATGCGATCACTCCGTTA
lacY-f2	5553	19	60.08	52.63	4.00	3.00	CATGTCTGCCCGTATTTTCG
lacY-r2	6916	20	59.69	45.00	4.00	2.00	GCATGTTCAATGCGATCACT

This output tells you where in your sequence the primer starts, the length in bases, the melting temperature in °C, the %gc (a measure of stability), the complementarity between either the primer and itself or the two primers (could allow them to make smaller products) and the 3' complementarity between a primer and itself or the two together (you want these to be below 2 or 3), and the sequence itself. I ordered some extra primers because sometimes they just don't work, and I wanted to be sure we had functional primers for each gene. In class you will be given a sequence handout, and you will spend time locating the boundaries for each gene and the locations of each of the primer sites based on the prep work outlined above.

General Procedures:

We will be using a technique called colony PCR today to generate template DNA for our PCR. The primers have already been resuspended (they are shipped dried down) and are at 10X concentration. We will be using a commercial 2X Taq master mix that has everything except template DNA and primers.

Protocol:

Generating template DNA

1. Take a loopful of cells from your plate, immerse the loop in 30 µl of 10 mM Tris in a 0.5 ml tube. Spin the loop to resuspend the cells (do for both your mutant and the 3110 positive control).
2. Mix the cells by pipetting up and down a few times.
3. Incubate the cells at 95°C for 5 minutes.
4. Use 4 µl of the resulting mix as the template in PCR.
5. We will need to amplify ~1 kb fragments of the *lacI*, *lacZ*, and *lacY* genes as this will give us appropriate PCR product sizes for sequencing. Because the *lacZ* gene is larger, we will amplify this gene in three different PCRs, while the *lacI* and *lacY* genes are only about 1 kb, so you will only need to do one reaction for each. The primer pairs we will be using are as follows:

For *lacI* use If1-Ir1

For *lacZ* use Zf1-Zr3, Zf3-Zr5, and Zf4-Zr2

For *lacY* use Yf1-Yr2

6. Each PCR cocktail should be prepared on ice and contain 25 μ l 2X PCR buffer with polymerase, 4 μ l template DNA, 5 μ l of the forward primer and 5 μ l of the reverse primer, and 11 μ l water. Be sure to do a PCR for both the W3110 wt control and your mutant for each gene.

Week 7 and Week 8 Agarose gel electrophoresis of PCR products and sequence analysis of mutant

Objectives and Overview

In these labs we will electrophorese our PCR amplification products (Week 7) using agarose gel electrophoresis techniques as described previously; and we will be looking at the sequences we have amplified to see whether we can determine why each strain is Lac⁻.

General Procedures:

Week 7 activities are similar to those previously used for plasmid DNA electrophoresis, except in this case we are electrophoresing PCR amplification products. Between the week 7 labs and week 8 labs PCR products were sent to the molecular core facilities at UT Austin to be sequenced by capillary sequencing methods. They sent us back the output files from the machine, so now we need to input those files into a program that will facilitate our analysis, along with control sequences we can obtain from Genbank for comparison.

Protocol:

These instructions are for analyzing one of your genes -- you should follow them for each of the genes you have readable sequence from. If you do not have readable sequences, you can use the sequences from a classmate. Please note that I can't possibly guess about everything that can go wrong with your sequences — so you'll have to use your wits or ask questions if you run into a situation that is not described below.

Analyzing your sequences

(note these directions were written using a Vista PC — XP and Mac users should be similar, but if you have problems please feel free to ask questions!)

Note to instructors: we have used Tlearn to deposit files for students, you can use any appropriate system or protocol to provide access of files to students

1. Go to the tlearn site and find the Excel file called "sequences.xls" in the Files/Lab folder. Find which sequences are yours by looking for your initials if you did your reactions alone, or your lab partnership initials if you worked with someone else. There will be at least two reactions for each PCR product. Next, write down which cell corresponds to your sequencing reactions (the cells are A1, B1, ..., H12).
2. Next, find the .scf files for your sequences in the Files/Lab/Sequences folder. They will be named by the cell you found in step 1.
3. Download the FinchTV sequence viewer application from <http://www.geospiza.com/finchtv.html>

4. Install and use the FinchTV application to view your forward sequences in the .scf files. You can use the sliders at left and bottom to adjust what you can see in your sequence trace — adjust these and scan your sequence to observe how much signal you have at different points along your sequencing run (why does it tail off?) Observe where you have clear, individual peaks in your run. At the top, you should see small histograms that describe the quality of your sequence read at each called base (if you cannot see these, click on the button with a Q and small histograms to reveal them). These histograms show what is called the phred score — essentially where the phred score is above 20 the base call is 99% accurate (see <http://www.phrap.com/phred/> if you are more interested in how phred works).
5. Open a text (use notepad or wordpad – not Word!) document for your sequence files and add in a fasta definition line by typing >description_of_sequence and then hitting return. The one important part of the definition line is that it starts off with a greater-than sign — your description can include spaces and odd characters, but most people try to stick to alpha-numeric characters to be interchangeable and make the sequence descriptors more easily machine-readable.
6. Go back to FinchTV and select the longest contiguous read of high-quality bases you can, copy these, and then paste them into your text file. You should now have something like this:

```
>my_name_for_my_sequence_YF1  
ATGGATTGATCTGCCATGCATG.....
```
7. Next open up one of your reverse sequence reads. You should reverse-complement this sequence by clicking on the button with the two arrows. Again, scan this sequence and then select the longest contiguous high-quality read and add it with a name to your sequence file. If you are looking at a *lacZ* read, follow this procedure for each of the forward and reverse sequences until you have all six in your file.
8. Acquire known gene sequences from Genbank to add to your sequence file (see the additional instructions at the end if you need help doing this). Save your sequence file as a text file with .fasta as an extension.
9. Download and install the Jalview application from http://www.jalview.org/Web_Installers/install.htm (you do not need to install a java virtual machine)
10. Start the Jalview application and then use the file>input alignment>from file to open your .fasta file. You should now see lines with all your sequences present in the window.
11. Try to manually align your sequences by shifting the sequences left and right as needed. You can select multiple bases and right-click them to cut, etc. To slide a sequence, hold down the shift key, left click on the base, and then use the pointer to move the sequence left and right. The search feature within a sequence is also helpful to try and find where sequences align. Another help may be to use BLAST to search the nucleotide database for a match that will show your alignment (instructions for BLAST can be found below).

12. When you have finished aligning your gene, create a composite copy of your gene by selecting the most 5' region you want include from your sequence, copying it, and then opening a text file and naming your sequence and pasting in what you just copied (sorry — I can't find an easier way to do this!). Next, add the next part of your gene by selecting it, copying it, and then adding it to your text file. When you have added in all your sequence information, save your text file as a .fasta file and then go back to Jalview and import the sequence. Once you have your sequence in Jalview, you can select it and then use the calculate>translate cDNA to get the peptide sequence.
13. From here on out, see what you can find out about your mutant! You can do a protein blast to look for dissimilarities, you can use a program such as SWISSMODEL or 3Djigsaw to thread your sequence onto a known structure (see me if you would like to do this)

Finding the *lacZ* gene in genbank

- go to <http://www.ncbi.nlm.nih.gov>
- in the search box, type lacZ
- this takes you to the Entrez combined database page where you can see that your search term can be found in thousands of articles and sequences. We want to find the gene sequence, so click on 'nucleotide' to start to see those records (2626 on 11/28/09)
- you may find that the sequence is not in the top results, though it is an *E. coli* gene. It can be hard to find what you want, but they are starting to implement better help features -- for example look at the right of the screen and you can see that we can easily look only in certain taxa. Click on '*Escherichia coli*' to get the 152 (on 11/28/09) results
- We want to find the gene sequence -- but none of the top hits give us that. There are a few ways to get to where we want to go from here, but let's try one of the top links provided from searching the 'Gene' database. Click on one of the lacZ from *E. coli* 0H157 links
- As you can see, this database has information on where the gene is located, the protein sequence, etc. On the left or at the very bottom there is a link to nucleotide -- click on the one at the bottom
- This takes us to the complete genome sequence! Where is our gene? Use your web browser's search feature to find *lacZ* on this page
- OK -- so now we know where it is, but do we have to search through the whole sequence to find bases 423606..426680 and then reverse-complement them?!?
- Luckily not -- click on the CDS link to the left (CoDing Sequence) and follow it to get the gene sequence
- This database record has lots of information for us, but the sequence is not in a friendly format with all the spaces and numbers. To get a more concise output, click on the drop-down 'Display' menu item at the top and select 'FASTA'

- There is not as much information with a FASTA-formatted record, but the top line '>info...' does have most of the relevant information, and the sequence data is very easy to manipulate. A FASTA-format is a very specific way to represent sequences so that databases can be inter-operable (the other sequence formats are specified as well, but a bit more cumbersome for some purposes)
- select and copy the entire sequence record, including the definition line (the >gi).

Using BLAST

We are now going to use our sequence to find other sequences in the database that are similar using an invaluable tool called BLAST (Basic Local Alignment Search Tool)

- go to <http://blast.ncbi.nlm.nih.gov/Blast.cgi>
- select 'nucleotide blast' from the basic blast options. This allows us to use a nucleotide query sequence to search against a nucleotide database. As you can see, there are many other flavors of BLAST that allow you to search nucleotide, translated nucleotide, an protein sequences against any of those databases.
- paste your sequence into the query sequence box and then choose nucleotide collection from the search set database drop down menu (we could search against human sequences, but you can do that on your own time!)
- click BLAST at the bottom and your search should be initiated
- there is again lots to go over with the output from a search, and the interface has been improved quite a bit from the early days. Basically you can see a top graphic of the best matches (hits) between your query sequence and those in the database. Red means a better match and the length of the bars indicates over what portion of your query the match extends. If you scroll down you can get a list of those hits, and then after the list you can start to see the alignments between your query and the database records (as a shortcut if you click on one of the bars in the graphic it takes you to this point on the page)
- the alignment output is what we will use when we get our mutant sequences back. If the query matches the database sequence, there is a vertical line between the two. The top hits will be perfect matches (after all, we used a sequence from the database, so it should match itself!), but if you scroll down you can begin to see that there are other imperfect matches and what those look like -- and this is how you can start to look for mismatches when you are using your own sequence as a query.
- if you would like, take the lacZ sequence and add in some letters in the middle (only A, T, G, C allowed!) and then BLAST it again to see what a discontinuous match looks like.

* next, on your own try to find the LacI protein sequence and do a protein BLAST to see what those results look like.

In the protein alignments, because amino acids can be chemically similar, the alignment has the single-letter amino acid in the middle if the query sequence is identical to the database sequence and a + if it is a similar replacement and space if the two amino acids are chemically distinct (scroll down through alignments to see sequence comparisons that look like this).