

- (12) 1. The DNA sequence of the *arc* gene is shown below. The promoter sequence is underlined and the coding sequence is shown in bold -- the translational start codon is ATG and the translational stop codon is TAA. Numbers above the DNA sequence indicate the distance from the transcriptional start site (number 1).

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5'  TGTATTGAC   ATGGATAGAAG   CACTCTACTA   TATTCTCAAT   1
                                     AAGTCCACGG
11  TGGACCTGTA   21  TTGTGAGGTG   31  AATATGAAAG   41  GAATGAGCAA   51  AATGCCGCAG
61  TTCAATTTGC   71  GGTGGCCTAG   81  AGAAGTATTG   91  GATTTGGTAC   101 GCAAGGTAGC
111 GGAAGAGAAT   121 GGTCGGTCTG   131 TTAATTCTGA   141 GATTTATCAG   151 CGAGTAATGG
161 AAAGCTTTAA   171 GAAGGAAGGG   181 CGCATTGGCG   191 CGTAAAGTTG   201 AAGC   3'

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Predict the expected phenotypes (Lac⁺ or Lac⁻) of *lacZ* operon fusions and *lacZ* gene fusions at the indicated site in the *arc* gene and briefly explain the rationale for your prediction in the following table.

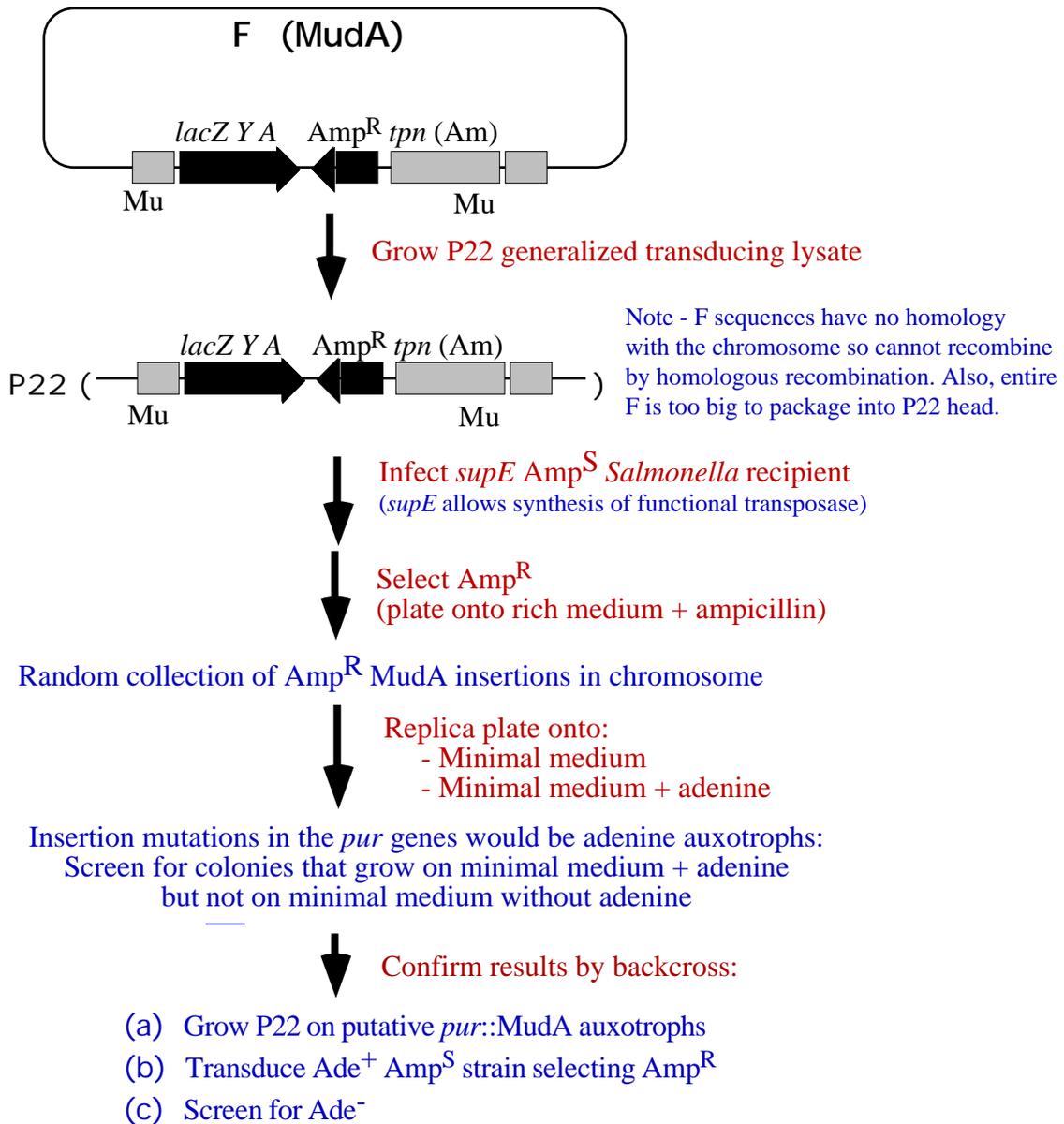
Position of insertion	LacZ expression?			
	operon fusion	rationale	gene fusion	rationale
39	+	if inserted in the correct orientation	+	Correct reading frame so: if inserted in the correct orientation
	-	if inserted in the opposite orientation	-	if inserted in the opposite orientation
40	+	if inserted in the correct orientation	-	Wrong reading frame so: if inserted in the correct orientation
	-	if inserted in the opposite orientation	-	if inserted in the opposite orientation
201	+	if inserted in the correct orientation	-	Untranslated region so: if inserted in the correct orientation
	-	if inserted in the opposite orientation	-	if inserted in the opposite orientation

- An insertion at position 39 refers to a fusion located immediately after position 39 in the DNA sequence.

- **Understanding the difference between operon and gene fusions is the key to answering this question. Expression of both operon and gene fusions demands that the fusion is oriented in the correct direction downstream of a transcriptional start site (i.e. a promoter). Expression of gene fusions also requires that the fusion is in the correct reading frame within an open reading frame (i.e. between the translation start site and the translation stop site).**

- (8) 2. MudA(*lac*, Amp) is a derivative of phage Mu that can form *lac* operon fusions but has amber mutations in the Mu transposase genes. Because Mud is 37 Kb and P22 packages about 44 Kb, Mud insertions can be moved between *Salmonella* strains by P22 transduction.

Given an insertion of MudA on the 100 Kb F-plasmid, draw a diagram showing how you could isolate MudA insertions in the *pur* genes required for adenine biosynthesis. Describe the strains you would use and any media needed.



- (10) 3. Wanner and colleagues isolated a large number of Mud(*lac*, *Amp*) operon fusions in *E. coli* that are induced by starvation for phosphate (designated *psi*). These mutants produce white colonies on high phosphate medium + Xgal but produce blue colonies on low phosphate medium + Xgal. The mutations mapped at many different positions on the *E. coli* chromosome.

They used one of these *psi* mutants to screen for insertions in genes that mediate the phosphate regulation. Questions about some of the resulting colonies are shown in boxes in the scheme below. Note -- this question involves hopping a *Tn5* transposon into a strain that already has a Mud operon fusion, then asking if the *Tn5* insertion affects the phenotype of the Mud fusion.

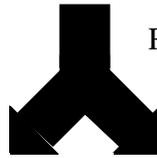
Infect *E. coli psiE::Mud1(lac, Amp)* recipient with Tn5-132(Tet) *cI O(Am) P(Am)*



Plate on rich medium with tetracycline

How would Tet^R colonies arise? [Where would the insertions be located?]

Because the λ cannot lysogenize (due to mutation in *cI*) or grow lytically (due to mutations in the *O* and *P* genes) any Tet^R colonies would be due to transposition from the λ onto the chromosome. The insertions would be random, resulting in insertions at different sites in each cell.



Replica plate onto:

Low phosphate medium + Xgal

High phosphate medium + Xgal

How would a colony that is blue on both replica plates arise?
[Where might such insertions be located?]

A Tn5-132 insertion that disrupts a negative regulator (e.g. a repressor protein) would be unable to repress the *psi::lac* fusion during growth on high phosphate medium and thus would express Lac constitutively.

How would a colony that is white on both replica plates arise?
[Where might such insertions be located?]

A Tn5-132 insertion that disrupts a positive regulator (e.g. an activator protein) would be unable to turn on the *psi::lac* fusion during growth on low phosphate medium and thus would not express Lac under either condition.

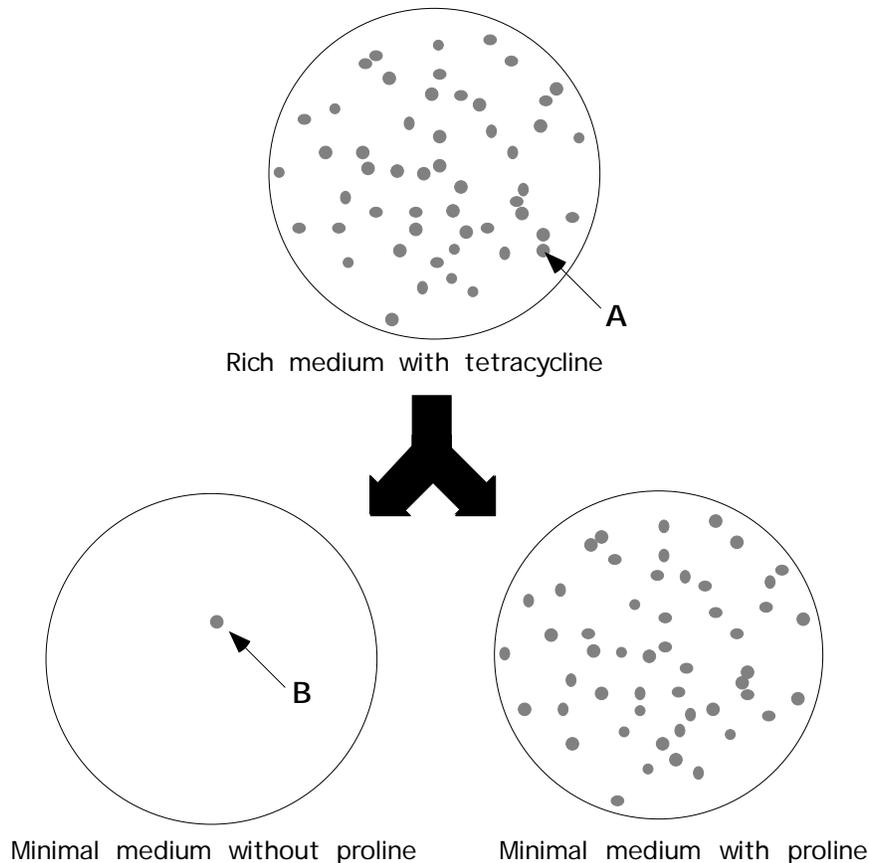
You could also get this phenotype from an insertion that prevents transcription of the *lacZ* gene in the *psi::lac* fusion.

Two ways this might happen are: (i) a Tn5-132 insertion in the *lacZ* gene or (ii) a Tn5-132 insertion located upstream of the *lacZ* gene that is polar on *lacZ* expression.

Hint: there is more than one explanation for colonies with the white-white phenotype. **Note that the target size for a regulatory gene is typically much larger than the region upstream of an operon fusion where you could get functional downstream expression from a P_{out}! Therefore, the constitutive blue mutants are very unlikely to be caused by such an insertion.**

- (6) 4. What is the rationale for using *cI O(Am) P(Am)* to deliver Tn5-132(Tet)?
Because transposition is a rare event, a way of selecting for transposition events is needed. The λ cannot lysogenize due to the mutation in *cI* or grow lytically due to the mutations in the *O* and *P* genes. Therefore, any Tet^R colonies must be due to transposition from the λ onto the chromosome.
- (12) 5. The *proBA* genes are required for biosynthesis of proline. To isolate a Tn10 insertion near the *proBA* genes, a strain with a nonsense mutation in the *proB* gene was transduced to

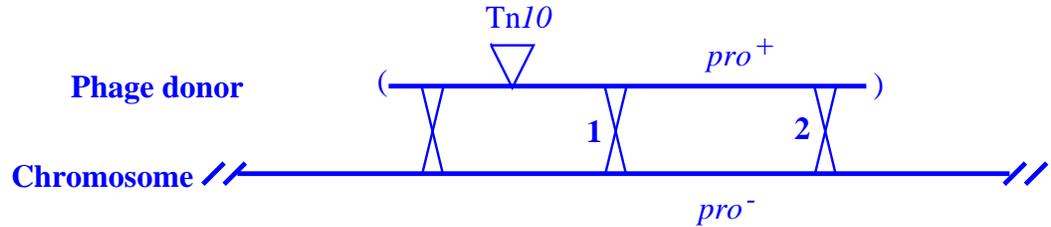
Tet^R with a phage lysate grown on a random pool of Tn10 insertions in the *Salmonella* chromosome. The Tet^R colonies were then replica plated onto minimal medium plus or minus proline. A diagram of the colonies observed on each plate is shown below.



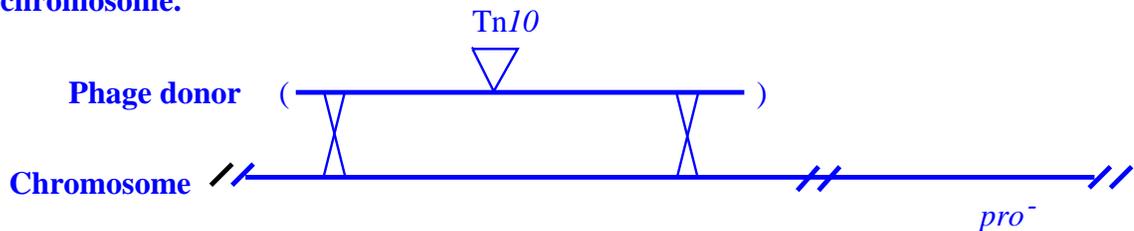
- What is the most likely explanation for the phenotype of the colony indicated by the arrow marked A?
This colony is unable to grow on minimal medium either with or without proline, indicating that it is an auxotroph due to inheritance of a Tn10 insertion that disrupts some other biosynthetic gene.
- What are two potential explanations for the phenotype of the colony indicated by the arrow marked B?
This colony has acquired the ability to grow on medium without proline. This is most likely to be due to either :
 (i) **co-transduction of *proB*⁺ with a linked Tn10 insertion (the desired result), or**
 (ii) **reversion of the amber mutation in the *proB* gene.**
- Diagram a genetic experiment you could do to distinguish between the two explanations for colony B. [Your diagram should describe the donor and recipient strains, any selections and screens required, and the media used.]

Backcross:

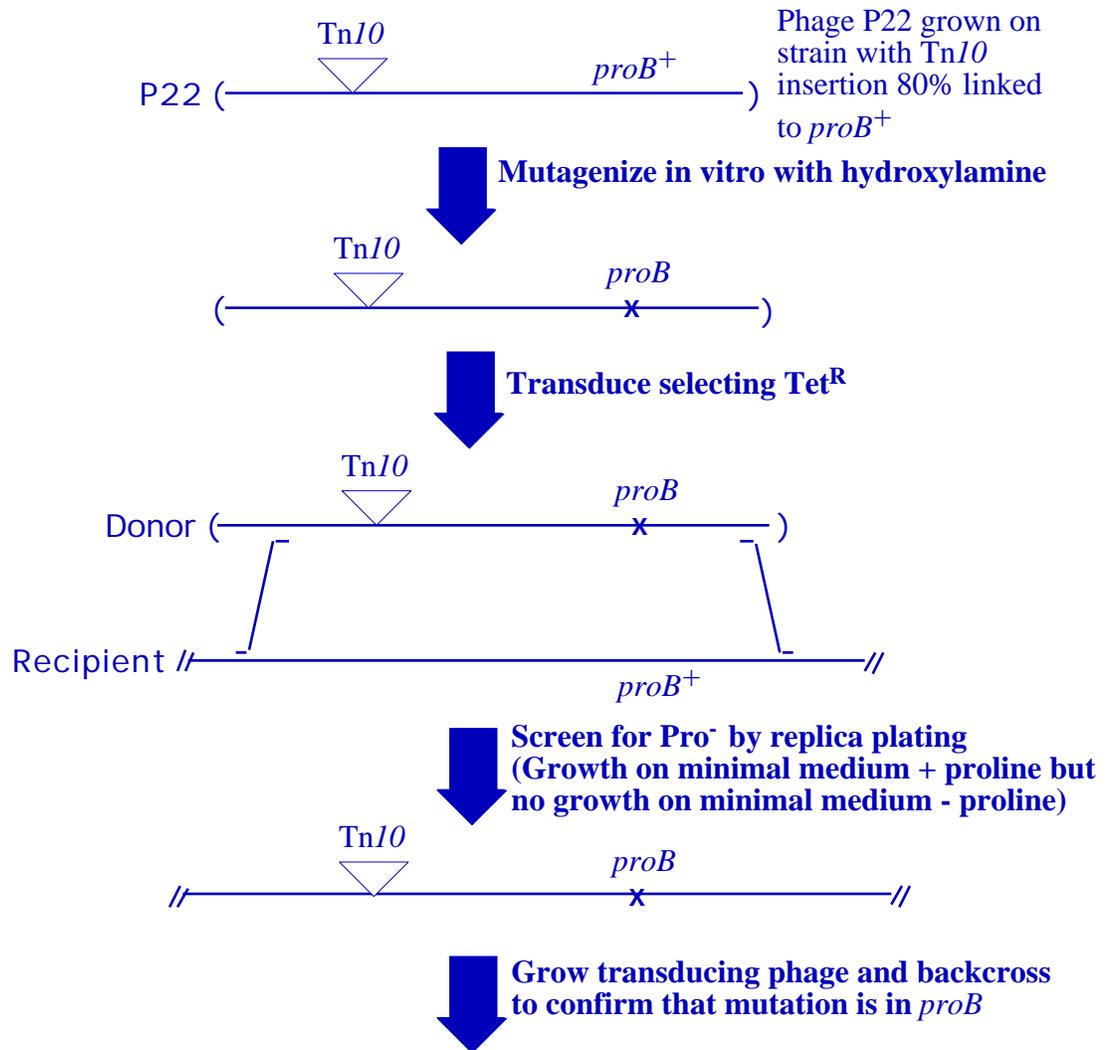
If the *Tn10* is linked to the *proB* gene then a single transducing particle can carry both the *Tn10* and the *proB*⁺ gene, so some of the Tet^R transductants will become Pro⁺ (crossover #2) and some will remain Pro⁻ (crossover #1).



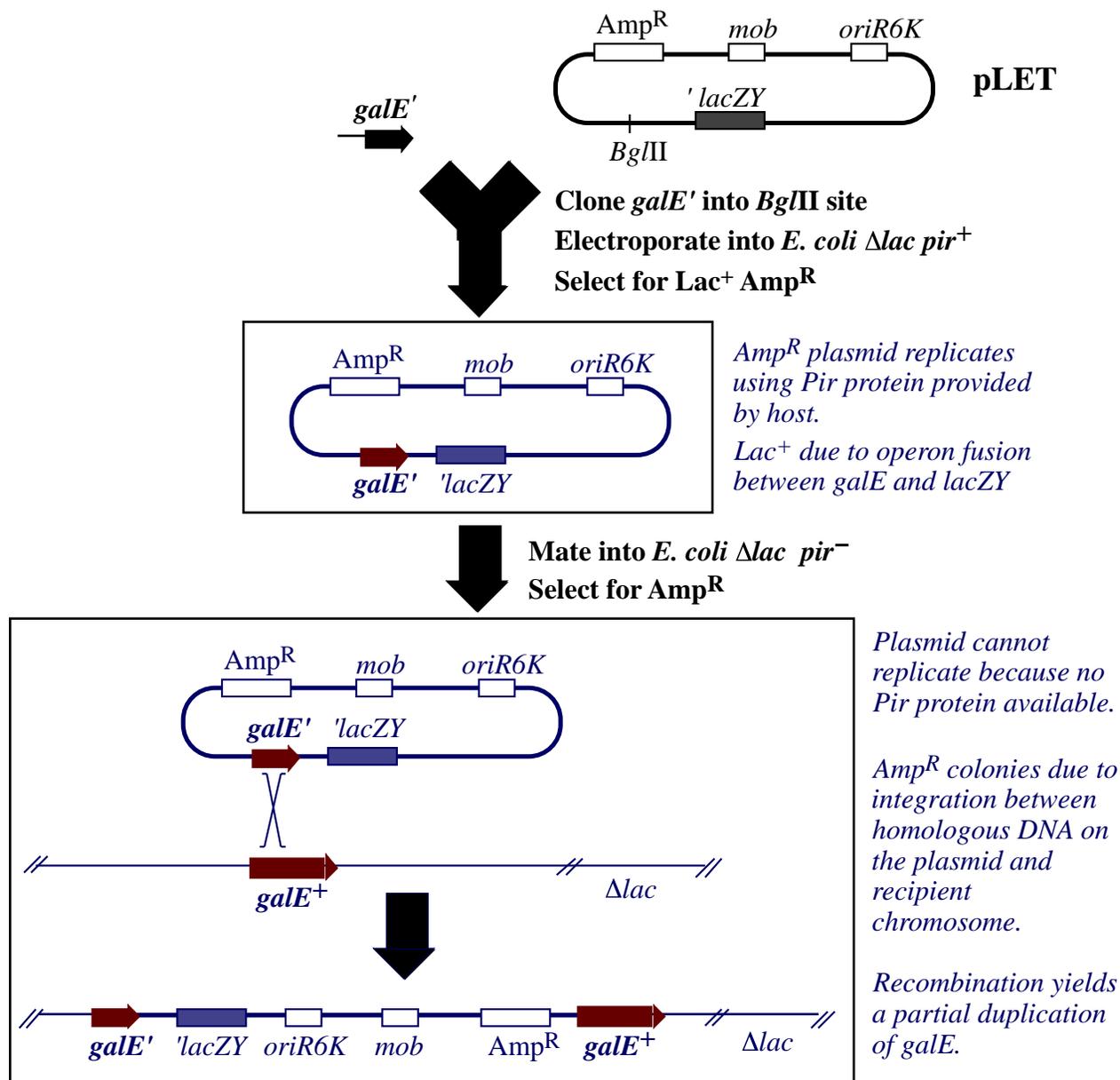
If the *Tn10* is NOT linked to the *proB* gene then all of the Tet^R transductants will remain Pro⁻ because a transducing particle cannot carry both regions of the chromosome.



- (10) 6. Draw a diagram showing how could you use a *Tn10* insertion 80% linked to the *S. typhimurium proB*⁺ gene to isolate point mutations in *proB*. [Your diagram should describe the donor and recipient strains, any selections and screens required, and the media used.]

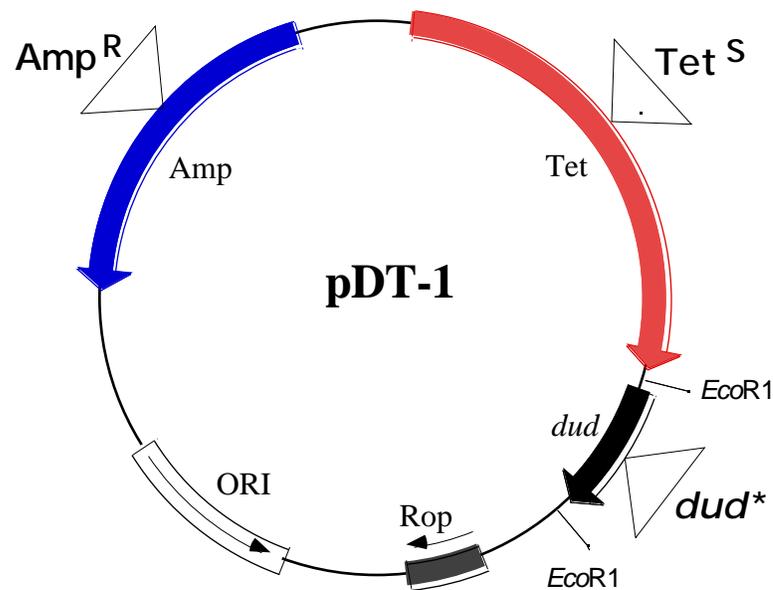


- (12) 7. A DNA fragment carrying the promoter and the first 660 base pairs of the *galE* gene from *E. coli* was cloned in front of the promoter-less '*lacZY*' genes in the vector pLET. The *oriR6K* origin on pLET requires the Pir protein for replication. In the boxes below, indicate the results expected for each selection. Draw a diagram showing any recombination events.



- (6) 8. Would the phenotype of the final strain from question #7 be Gal⁺ or Gal⁻? [Explain.]
Gal⁺ because the integration event yields one truncated copy of *galE* and one wild-type copy of *galE*, both with their own promoters intact.
- (6) 9. Mutations in the *S. typhimurium* *dud* gene result in extreme sensitivity to the dye alizarine yellow in rich medium. The *dud*⁺ gene was cloned into an Amp^S Tet^R plasmid vector for site-directed mutagenesis by the *mutS* method. Using mutant oligonucleotides that anneal

at the position marked with the triangles, the following dud^- Amp^R Tet^S derivative was constructed.



- Describe a definitive genetic approach to prove that the Dud^- phenotype is specifically due to the site-directed mutation [i.e., without DNA sequencing].
Site-directed reversion of the mutation to the original, wild-type sequence.
[Note -- spontaneous reversion is not an acceptable answer because you couldn't distinguish a true revertant from a second site revertant]
 - Why would this approach be better than simply selecting for spontaneous revertants?
Spontaneous reversion could be due to second-site suppressors as well as true-reversion. Furthermore, it would be possible to obtain revertants even if the mutation was at some site other than the predicted position.
 - Describe the general features of the mutant oligonucleotide(s) and recipient strain would you use for this experiment.
Three mutant oligonucleotides:
 - (i) Amp^R to Amp^S oligonucleotide**
 - (ii) Tet^S to Tet^R oligonucleotide**
 - (iii) dud^* to dud^+ oligonucleotide****Transform into a Amp^S Tet^S dud^- recipient strain , select for Tet^R and screen for Dud phenotype and Amp^S.**
- (6) 10. In the *dut ung* method of site-directed mutagenesis, single-stranded template DNA is purified from a *dut ung* mutant, a mutant oligonucleotide is annealed to the single-stranded template DNA, the second complementary strand is synthesized, then the resulting double-stranded plasmid DNA is transformed into a $dut^+ ung^+$ recipient.

- Why does this enrich for the desired mutant plasmids?
In many of the plasmids, the template strand will contain U residues which are excised in the *ung*⁺ recipient, resulting in the inability to replicate this strand of DNA.
In contrast, the newly synthesized strand lacks U residues and thus is replicated in the *ung*⁺ recipient,.
- This is an enrichment, not a selection. Typically 50% of the resulting colonies do not have the desired mutation. Suggest one way that such wild-type colonies might survive in the *dut*⁺ *ung*⁺ recipient.
Two potential reasons include:
 - mismatch repair may occur before replication of the transformed plasmid -- if the mutant nucleotide is repaired to match the template strand then the template sequence will be present in the replicated plasmid;**
 - some of the template plasmid molecules may not have incorporated sufficient U residues in the *dut ung* parent to prevent DNA replication.**

- (12) 11. The *lacY* gene encodes a permease that transports lactose. The *lacY* gene has four histidine codons. Using site-directed mutagenesis, Kaback and colleagues made nucleotide substitutions in each of these histidine codons. The properties of the resulting mutants are shown in the table below.

	Mutation(s)	Lactose binding ¹	Lactose transport ²
a	None	+	+
b	His ₃₅ -Arg	+	+
c	His ₃₉ -Arg	+	+
d	His ₂₀₅ -Arg	+	+
e	His ₃₅ -Arg His ₃₉ -Arg His ₂₀₅ -Arg	+	+
f	His ₃₂₂ -Arg	+	-
g	His ₃₂₂ -Asn	+	-
h	His ₃₂₂ -Gln	+	-
i	His ₃₂₂ -Lys	+	-

¹ + indicates the K_M for lactose binding to the permease is unchanged.

² - indicates that the permease is unable to catalyze active transport of lactose into the cell.

Using the attached codon table and amino acid wheel, answer the following questions about these results.

- What is the simplest explanation of these results? [Your answer should account for both the lactose binding and lactose transport results for each mutation.]
 - Lactose binding indicates that the protein is properly folded despite the mutation changes;**
 - His₃₅, His₃₉, and His₂₀₅ are not directly involved in catalysis of lactose transport (or possibly Arg substitutions are simply permissive, but that is unlikely);**

(iii) His₃₂₂ plays an important role in catalysis of lactose transport but not lactose binding per se.

- Why were His-Arg substitutions used to test the importance of the His residues?
The simplest reason is that this change only requires a single bp substitution.
- Why were the His-Asn, His-Gln, and His-Lys substitutions also tested for His₃₂₂?
[Why bother testing additional substitutions and why test these particular substitutions?]

These residues have different charge or size to determine if the effect is the same as for the Arg substitution. The same effect from multiple amino acid substitutions argues that the effect is not simply because one particular amino acid is not permissive at that site in the protein.