

Cow of the Future: Genetically Engineering a Microbe to Reduce Bovine Methane Emissions

by

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Part I – The Proposal

A wealthy cattle baroness has hired you! You don't know much about cattle, so what exactly is your job? Read on to find out! You will get to know a small cast of characters including the Baroness, Dr. Kenmore (a microbiologist), and an amazing bacterium called *Methylococcus capsulatus*. Your job will be to help the Baroness understand and evaluate Dr. Kenmore's "Cow of the Future" project proposal.

Cast of Characters

The Baroness: The Baroness is a self-made billionaire who would like to be a leader in the cattle industry by finding ways to make it more environmentally friendly. In particular, one of her immediate goals is to reduce the large amount of methane emitted into the atmosphere by the millions of cattle in her extensive herds. Some progress in methane reduction has been made by scientists who adjusted the composition of cattle feed (Rathke, 2013), but the Baroness believes that much more could be done by adopting more cutting-edge approaches. Toward this goal, she has established a foundation to fund research projects that could provide new solutions to problems in the cattle industry.

Methylococcus capsulatus: This Gram-negative obligate aerobe can metabolize methane (so we call it a "methanotroph") (Ward et al., 2004; Chistoserdova, 2011). One well-characterized strain of *M. capsulatus* was isolated from the Roman baths of Bath, England (so it is referred to as *M. capsulatus* [Bath]). The complete genome of this microbe has been sequenced: 3,304,697 base pairs containing genes encoding an estimated 3,000 proteins (Ward et al., 2004). Some of these proteins are enzymes that allow *M. capsulatus* to metabolize methane, using it as a carbon and energy source. One particularly important enzyme, comprised of three polypeptides, is called particulate methane monooxygenase (pMMO). pMMO catalyzes a reaction between methane and oxygen that produces methanol (Chistoserdova, 2011). This reaction is the first step in a pathway that allows *M. capsulatus* to convert methane into metabolites (Figure 1, next page) that become amino acids and other components of new cells.

Dr. Kenmore: This highly respected microbiologist has been studying *M. capsulatus* for more than twenty years in his laboratory in the Animal Sciences Department of a major university. Recently, he cloned the *M. capsulatus* genes encoding the enzyme methane monooxygenase (*pmoC*, *pmoA*, and *pmoB*) and expressed them in *Escherichia coli*. Dr. Kenmore is a big believer in probiotics—he eats yogurt and kefir every day. One of his research projects is investigating the possibility of using genetically engineered bacteria as probiotics to accomplish a variety of goals.

Cattle and their rumenal microbiota: Approximately 23% of methane emissions in the U.S. are from cow burps ("enteric fermentations"). This number is troubling because the Global Warming Potential (GWP) of methane is 21 times that of carbon dioxide on a 100-year time scale (Environmental Protection Agency, 2013). The following is a little background that explains how and why cows burp so much methane and how microbes are involved. Cows and related animals have a more complicated digestive system than humans. It includes a compartment called the rumen that food passes through before reaching the stomach. The rumen is like a big fermentation vessel. It provides a perfect environment to support the growth of large, diverse populations of microbes: it is large, has a constant temperature (39°C), constant pH (in the range of

5.5 to 7), and provides a continuous flow of nutrients (in) and waste products (out). The rumen is filled with bacteria and protozoa, some of which degrade cellulose. That's why ruminants, unlike many other animals, can digest cellulose—the microbes do it for them! Food remains in the rumen for about 10 hours, during which time the resident microorganisms hydrolyze ingested cellulose to glucose. Rumenal bacteria then use this glucose as a substrate for fermentation, producing volatile fatty acids and gas including methane. Fermentation in the rumen generates enormous quantities of gas: 30–50 liters per hour in one adult cow (Bowen, 2009), about 25% of which is methane produced by methanogens (Moate et al., 1997). Belching is how ruminants continually get rid of fermentation gases. Dairy scientists use the fancy term “eructation” to describe this release of gas produced by enteric fermentations occurring in the rumen. And this is where all that bovine methane is coming from (Bowen, 2009).

Dr. Kenmore's Cow of the Future Project

Seeking funds to support his research, Dr. Kenmore submitted a proposal to the Baroness' foundation. You are going to help the Baroness evaluate this proposal. From lectures and and from researching answers to the questions in this case, you will learn about the science in the Kenmore Project. You will need to explain numerous aspects of this project to your employer, the Baroness. She needs to know whether the Kenmore Project is actually possible and whether it is fundamentally sound from a scientific perspective.

Figure 1 summarizes the methylotrophic (methane-utilizing) metabolism of *M. capsulatus*. Don't worry about the details of this diagram, but it will help you to understand some aspects of Dr. Kenmore's Proposal.

Here is a synopsis of the Kenmore Proposal:

Principal Investigator: Charles Kenmore, Ph.D.

Proposal Title: A Novel Probiotic Approach for Reducing Bovine Methane Emissions
Submitted in response to RFP1: Cow of the Future

We propose a plan for a Cow of the Future project to address the significant contributions of cattle to rising levels of methane, a greenhouse gas that is produced in large amounts as a metabolic waste product by microbes residing in the rumen of cattle. We describe a probiotic strategy in which a methane-consuming bacterium (a methanotroph, Figure 1) will be added to cattle feed. Following the principles of probiotic therapy, the ingested methanotroph will colonize the rumen of cattle, where it will grow and metabolize the methane produced there by rumenal bacteria. The methanotroph will convert methane to biomass that will:

- benefit the cattle nutritionally;
- significantly reduce the amount of methane emitted by the treated cattle.

Naturally occurring methanotrophs cannot survive in the rumen, so we will create one that can survive in this environment by using recombinant DNA strategies. To accomplish this, we will take the genes encoding methane

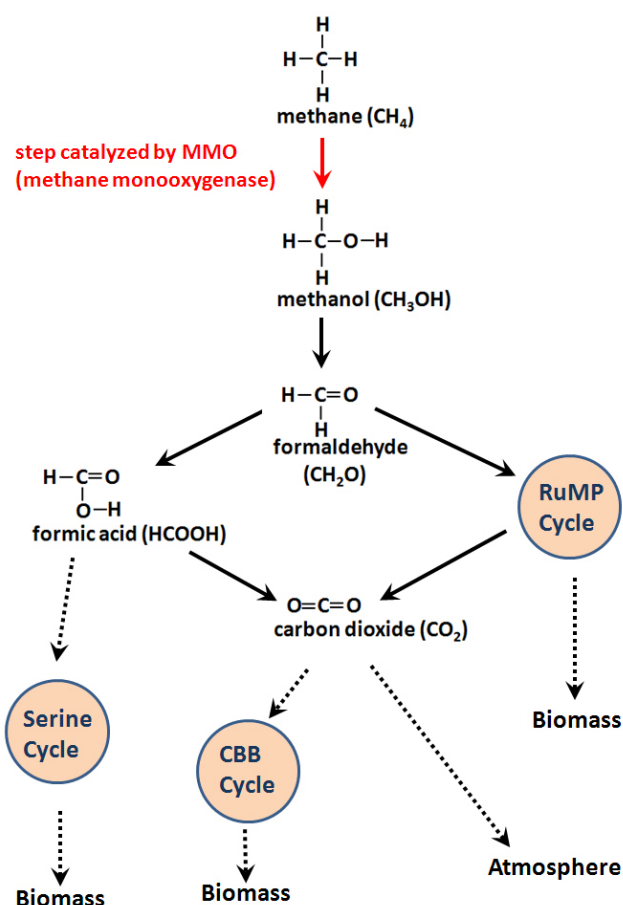


Figure 1. Metabolic pathways that enable methylotrophic metabolism in *Methylococcus capsulatus*. Each solid line depicts a chemical reaction catalyzed by a specific enzyme. For example, the first step of this pathway is catalyzed by particulate methane monooxygenase. Dashed lines represent processes involving multiple steps and multiple enzymes, some of which are not yet well understood. CBB denotes the Calvin Benson Bassham cycle; RuMP is the ribulose monophosphate pathway. This figure is based on Fig. 2 in Chistoserdova et al. (2005).

utilization enzymes from the bacterium *Methylococcus capsulatus* and move them into a well characterized, safe (non-pathogenic) laboratory strain of *E. coli* (*E. coli* Nissle) that has been used previously as a human probiotic (Mutaflor®) and that can colonize the cow rumen. As proof-of-principle that this approach is feasible, we propose a pilot study in which the genes encoding the enzyme particulate methane monooxygenase (*pmoC pmoA pmoB*) will be incorporated into a plasmid and then this plasmid will be introduced into *E. coli* in the laboratory using the process of transformation. This recombinant *E. coli* will be fed to cattle so that it can colonize the rumen. In the rumen, the *E. coli* will grow and produce particulate methane monooxygenase that will metabolize much of the methane produced there by rumenal microorganisms, converting it to methanol. If these initial studies are successful, we will perform more extensive genetic engineering of *E. coli* to introduce the full methane-utilization pathway of *M. capsulatus* into *E. coli* Nissle.

The plasmid directing expression of the methane utilization pathway will be lost from *E. coli* unless we provide selective pressure to force its continued maintenance when our recombinant bacteria are colonizing the cow rumen. Therefore, we have included a selectable marker on the pMMO expression plasmid: the *blaA* gene (encoding β -lactamase) will allow us to use ampicillin resistance as a selectable marker. Thus, the antibiotic ampicillin will be added to cattle feed along with the recombinant *E. coli*. We anticipate that we can use these two simple additions to cattle feed (recombinant methanotrophic *E. coli* and ampicillin) as an effective and inexpensive approach to significantly reduce bovine methane emissions.



References

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Part II – The Underlying Science

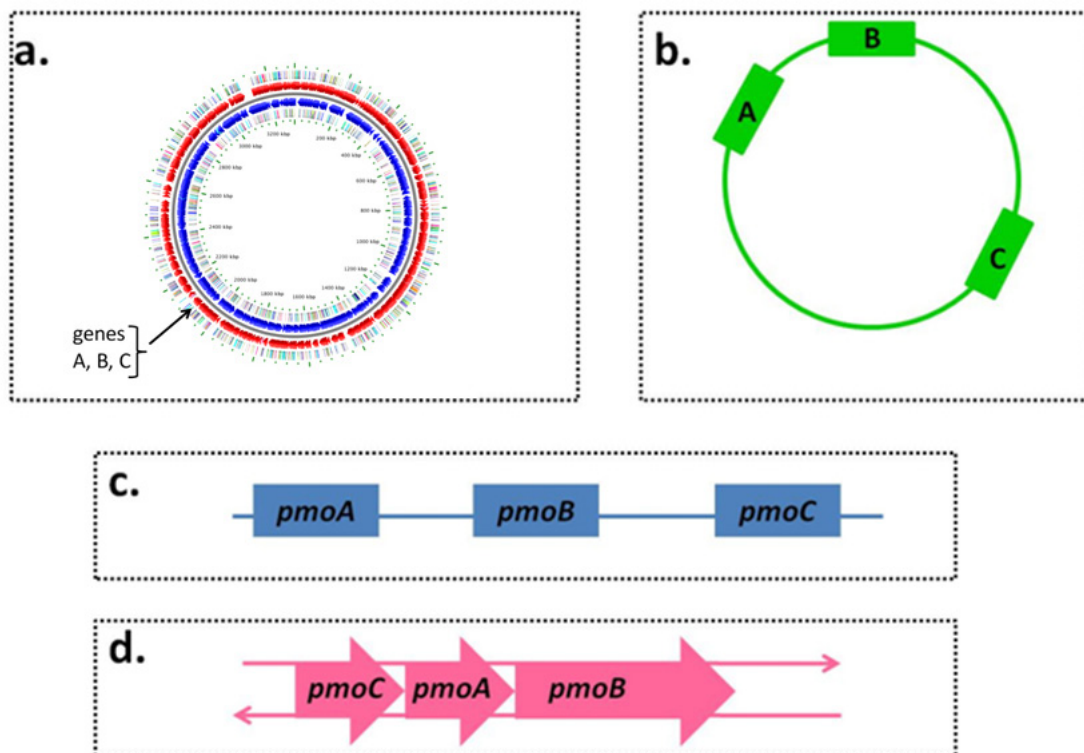
The Baroness will arrive next week to hear a presentation from you explaining the science underlying Dr. Kenmore's probiotic project. Remember that she ultimately needs to know: *Is the Kenmore Project actually possible? Is it fundamentally sound from a science perspective?* The enzyme pMMO (particulate methane monooxygenase) is a key part of his project, so you will need to understand some basic features of this protein complex and the genes encoding it. You review the literature about pMMO and learn that pMMO is a membrane bound copper and iron containing enzyme. The structural genes for this enzyme lie in a three-gene operon named: *pmoCAB*. This operon codes for the three integral membrane polypeptides: PmoC, PmoA and PmoB of approximately 23, 27, and 45 kDa, respectively, that make up the final functional enzyme pMMO (McDonald et al., 2008). It is really important that the Baroness understand that the genes contain all the information necessary for a cell to produce methane monooxygenase. This is the reason that genetic engineering will work!

You decide that the Baroness must understand the answers to the following specific questions to be able to understand the Kenmore Project and to be able to answer her general question about its feasibility.

Questions

- How are the *pmoC*, *pmoA*, and *pmoB* genes arranged in the DNA? You enlist some help from a college freshman to draw the figures (Figure 2). Which would you choose as the most useful and accurate depiction of the arrangement of the *pmoC*, *pmoA*, and *pmoB* genes in the chromosome of *M. capsulatus*? Give reasons to support your choice.

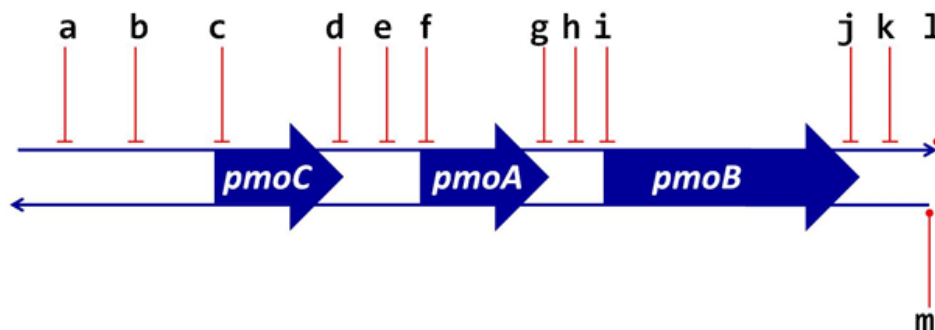
Figure 2. *pmoCAB* operon of *Methylococcus capsulatus*.



2. A gene includes a number of important sequences that ensure transcription and translation of the gene: promoter, initiation and termination sequences, RBS, start and stop codons. What is the role of each of these? For each of these, briefly describe its function (be sure to indicate whether it is important for transcription or for translation) and as the Baroness may question why she must understand such detail, indicate whether you think it would be important to include it in your explanation to the Baroness.
3. Where are the important sequences from Question 2 above located? An image will be very helpful to show this. First you need to figure out the answers to the following:
 - a. How many promoters would be necessary to allow transcription of the *pmoCAB* operon?
 - b. How many mRNA molecules will be produced when this operon is transcribed?
 - c. How many ribosome binding sites will be present on the RNA produced when the *pmo* operon is transcribed?
 - d. How many start codons and stop codons will be present on the RNA produced when the *pmo* operon is transcribed?

The undergrad in the lab draws this diagram (Figure 3). Now you have to add the labels to the lettered sequences marked on the DNA.

Figure 3. Depiction of the *pmo* operon.



- e. Which lettered area best marks the area within the DNA sequence where RNA polymerase would bind to ensure transcription of *pmoA*, *pmoB* and *pmoC* as a unit?
- f. Which lettered area (or areas) best marks the area within the DNA sequence where you would find a site that serves as the initiation site for transcription?
- g. What about for termination of transcription?
- h. Which lettered area (or areas) best marks the area within the DNA sequence where you would find sequences encoding a translational start site? What about the location(s) of DNA sequences encoding translational stop codons?
- i. Which lettered area indicates the 5' end of a DNA strand?

References

McDonald, I.R., L. Bodrossy, Y. Chen and J. C. Murrell. 2008. Molecular ecology techniques for the study of aerobic methanotrophs. *Applied and Environmental Microbiology* 74 (5): 1305–1315.

Part III – A Mistake?

Cloning pMMO genes in *E. coli* will allow *E. coli* to produce the methane monooxidase that oxidizes methane to methanol, the first step to converting the methane to biomass. You want the Baroness to understand that taking the DNA from *M. capsulatus* and cloning it in *E. coli* will result in *E. coli* producing pMMO enzyme that is identical to that produced in *M. capsulatus*. It is all in the Central Dogma! The easiest way to show this is to look at the sequence of DNA and compare it to the sequence of amino acids in the protein.

Again the college freshman helps you. You instruct the student to get the gene sequence for *pmoC1* from the National Center for Biotechnology Information (NCBI) site at: <http://www.ncbi.nlm.nih.gov/nucore/14456718> and prepare a set of slides for you to show how the gene will be transcribed and translated to make a protein. Here is the sequence that the student has prepared for your presentation:

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1      GTCGACTGGG CACCAGCCGG ATGCGTCCGT CAACCCCGAC TGTTCGCCA AGAACTCCGG
61     CCTTCTGTGG CGGGATGACG TTGAGCACAT CGGCACAGAA AGTGTGGAAC TCGGTTTCGA
121    CGGTTTCGCCC GCGGCATCG ATCCGCTCCA GCCGTCCCTC TTTTTCGAG GAAACCCATT
181    CCACCATGTC TCCGTAGAGT TCGCGCCAGG CCTGCTCGAA TGCCGGCTGC TTGGAAAACC
241    GGGTCTTGGC ATCGAGTATC AGGATCTTGG AGCGGGGTTT GTGCTTTTTT ATGTAATAGG
301    CGATGAGCGA GCGCGTTCG TAGGGGCCAG GCGGACAACG ATAGGGAGAA GGTGGTGCGG
361    TGATCAGAAC CAGGCCGCCG TCGGGCATCG CGCGGATCTG CCGGGCGAGC AAAGCGGTCT
421    GGGGGCCGGC CTTCCAGGCA TGGGGAACGA ACCGGCTCGC CGCCTCGTCA TAGCCATTA
481    TCGCCTCCCA GCGAAAGTCG ATGCCGGGGG AGAGGACTAG CCTGTGCTAG GTGACCTCGG
541    CACCATCGTT CAGTATCACA CGCCGTCGCT GCCGATCCAG GCGGGCGACC CGGGCGGTTA
601    CCTTTCCTAT ATCCAGCTCC CGCCGCAACC AGTCATAAGA CCGCGCGAGA GTCCCCATGT
661    CCCCAGAGCC GCGACTGCT TCGTTCGATC CGGGGCGAGG AAGATAAGTC TCCTGCGGTT
721    CGATCAATGT GATCGTCAGA CCGGGATTCA TCTGCTTCAG ATAGCGGGCG GCCGTGGCAC
781    CGCCATAACC GCCACCGACG ACCACGACCG GCCGCCTGAG CGAAGACCTT GCCGGAAGCG
841    CAGCCACCCA GTCCCAGCCC ATGCCGCGA CTGCCAGCAG GCGCAGGAAC CGCCGCCGGC
901    GGATCATGGC CTCTTTCCCA GAAAGCCGGC GATCGCTTCA ATGTCCTGGT TCGTGAGTCC
961    GGCAGCGATC CGGTTTCATGA CCGTGCCCGA TCTTTTTTCT TCACGGTACT CCCGCAACAG
1021   AGATGCCATC TCCTTCGCAT CGAAGCGGCG TAACGATGCC GGTTCGGGAA TCTGCTCCTC
1081   CTCGTCGGCA TGGCAGCCGA GGCAACCGAG CGCAGCCAAA ACCATGTCCG GTTTTTTCGGC
1141   CCGGGCCGGG AAACAGAGAG CGACAGCGAT CGTACCGATC TGGACGCGTC TCACAAAACGA
1201   CAAAACGTCA CGATGGGTGT TCGGTAGCTG AGTCACGGGG ATTTGTAGAA GTATAGGACC
1261   GACGGATTTT ATGCAAGCAT GTCGCTTTGA CCAAGCCGGG ATTCATGGA AGGGATGTCA
1321   TCGGGAGAGT TATTTATGTC GTTGATTTAT AAGAACTAC CCCTGCGTCA AAATGTGCGA
1381   GATTTTTTCT GACAGTTTGG GGGAGGGTGA TAGATCCTCC ACCGATGGAC CGGTACCGCC
1441   TCTGTTGCGG GGTCCATGAA ATGCCCGTTA GAGGCAGAAC CGATAGGGAA TTAGAGAAGC
1501   GGGCGTCGGC GCCGAATGCC GGCCCTGTC AACCATCACT TTAGGAGGAA CAAACAATGG
1561   CAGCAACAAC CATTGGTGGT GCAGCTGCGG CGTAAGCGCC GCTGCTGGAC AAGAAGTGGC
1621   TCACGTTGCG ACTGGCGATT TACACCGTGT TCTACCTGTG GGTGCGGTGG TACGAAGGTG
1681   TCTATGGCTG GTCCGCCGGA CTGGACTCGT TCGCGCCGGA GTTCGAGACC TACTGGATGA
1741   ATTTCTGTA CACCGAGATC GTCCTGGAGA TCGTGACGGC TTCGATCCTG TGGGGCTATC
1801   TCTGGAAGAC CCGCGACCGC AACCTGGCCG CGCTGACCCC GCGTGAAGAG CTGCGCCGCA
1861   ACTTCACCCA CCTGGTGTGG CTGGTGGCCT ACGCCTGGGC CATCTACTGG GGCGCATCCT
1921   ACTTCACCGA GCAGGACGGC ACCTGGCATC AGACGATCGT GCGCGACACC GACTTCACGC
1981   CGTCGCACAT CATCGAGTTC TATCTGAGCT ACCCGATCTA CATCATCACC GGTTTTTCGGG
2041   CGTTCATCTA CGCCAAGACG CGTCTGCCGT TCTTCGCGAA GGGCATCTCG CTGCCGTACC
2101   TGGTGTGTTG GGTGGGTCCG TTCATGATTC TGCCGAACGT GGGTCTGAAC GAATGGGGCC
2161   ACACCTTCTG GTTCATGGAA GAGCTGTTTC TGGCGCCGCT GCACTACGGC TTCGTGATCT
2221   TCGGCTGGCT GGCCTGGCC GTCATGGGCA CCCTGACCCA GACCTTCTAC AGATTCGCTC
2281   AGGGAGGGCT GGGCAGTCG CTCTGTGAAG CCGTGACGCA AGGCTTGATC GCGAAATAAG
2341   GAGCTTGTC A GGCTGGCGGG CCGGTTTTTT CGGGGTCTG GCGACAGGCC CCGGGGAACC

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You decide to give the Baroness and her guests cards printed with all of the codons and their corresponding amino acids. As you go through the sequence, each person will bring their codon card to the front of the room and show how the DNA and mRNA sequences encode a protein with a specific sequence of amino acids. This will be great, you think! You begin reading the sequence at site 1557, which is the position that GenBank indicated as the first nucleotide of the start codon of *pmoC1*. As you are underlining the sets of three nucleotides and discussing how each corresponds to a codon in the mRNA and an amino acid in the protein, you read ahead and notice that your student helper must have made a mistake!

You think, “There is a mistake in the DNA sequence! *Darn it!* Is this going to change the protein product? Will my audience notice? What a mess.”

You need to determine if the helper made a mistake. One quick way to do this is to use a BLAST search to compare the student’s sequence to the sequence that is actually in the database. Go to <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Use the Specialized BLAST option: Align two (or more) sequences using BLAST (bl2seq). Copy the DNA sequence above (student’s sequence) and paste it into the box designated as the Query Sequence (don’t worry about the numbers or about “tidying up” the sequence by removing spaces, BLAST will do this for you). Find the correct sequence for the *pmoCAB* operon from <http://www.ncbi.nlm.nih.gov/nucore/14456718>. Copy this sequence and paste it into the Subject Sequence box on the bl2seq form. Run the BLAST comparison (click the BLAST button) and note any differences/non-alignment.

Now you are ready to go to the next set of questions.

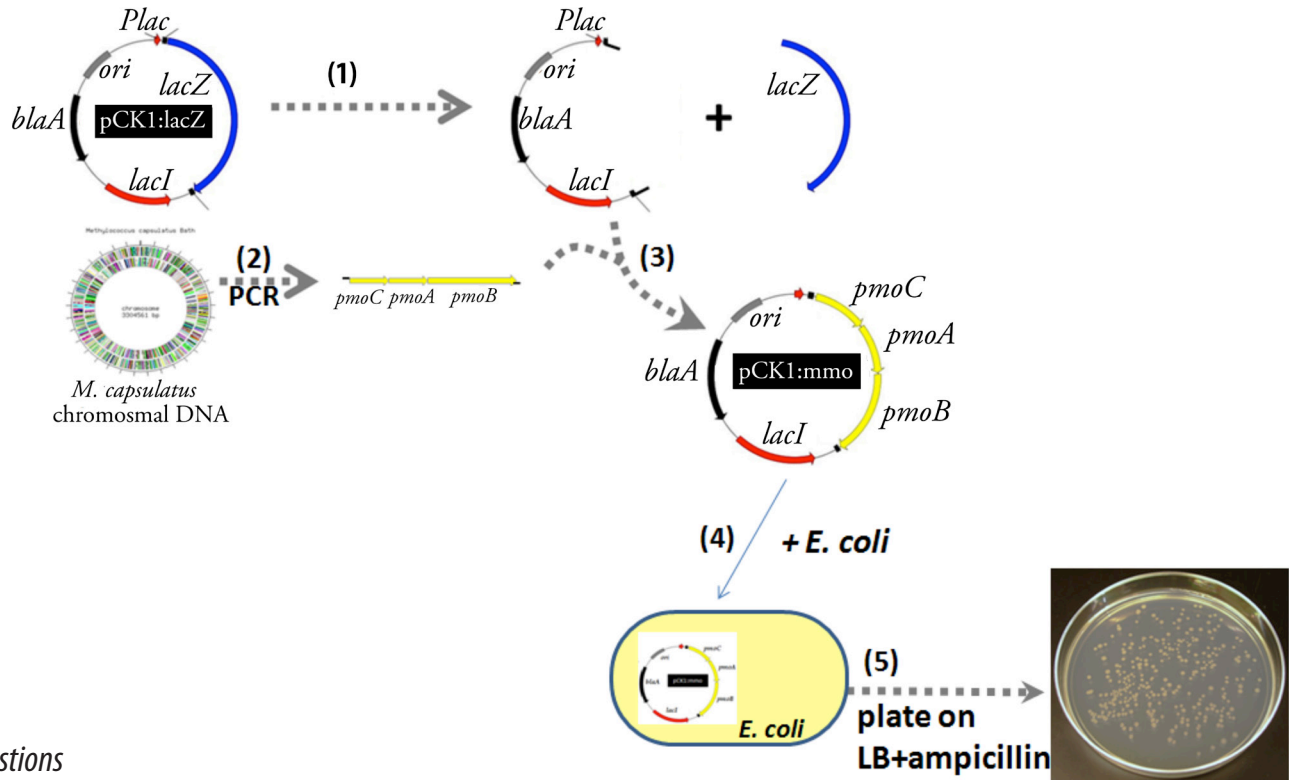
Questions

1. *Identification of the mistake.* If the nucleotides in the gene were changed as indicated in the mistake sequence, what type of mutation would this be? Explain.
2. *The DNA sequence shown is the sense (+) strand of the double stranded DNA that makes up the pmoC gene, encoding PmoC.* What would the mRNA sequence be when the template (-) strand DNA is transcribed? (Give first 60 bases). What would the amino acid sequence be when the mRNA is translated? (Give first 15 amino acids).
3. *Analysis of the data.* How is the protein product of this DNA sequence different from the PmoC that would be made if the gene did not have a mutation? Make a prediction as to the functionality of this altered protein compared to *PmoC*.

Part IV – The Cloning Process

The Baroness has now seen the *pmoCAB* operon and understands the basic mechanisms of DNA replication, transcription, and translation. Now you are ready to explain the cloning process proposed by the Kenmore lab that will create the plasmid that allows *E. coli* to express these genes! You have drawn a really nice diagram to show the process. Your goal is to explain why each labeled section is important.

Figure 4. Diagrammatic depiction of the cloning scheme.



Questions

Use the information in Figure 4 to address the following questions.

- Analysis of the cloning vector.* First you point out the plasmid vector: pCK1::lacZ. What is the function of each labeled section? How will each section be important in your cloning experiment?
- Review of the cloning process.*
 - During which step in the diagram would DNA ligase be used (1, 2, 3, 4, or 5)?
 - During which step in the diagram would a restriction endonuclease be used?
 - During which step in the diagram would the ampicillin resistance gene be important?
 - Which step represents the transformation of a bacterial cell?
 - What must be added to the growth media to ensure expression of the *pmoCAB* operon?
- Genetic exchange can occur naturally between bacteria by three different mechanisms.* Once in the cow, each of these mechanisms could result in the transfer of pCK1::mmo from the recombinant *E. coli* to a bacterium resident in the cow rumen. Explain how each mechanism would occur.



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