Use of Bioinformatics to Investigate β-Lactamase, a Mediator of β-Lactam Antibiotic Resistance

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Contents

Introduction

Bacterial antibiotic resistance is a major problem affecting health care today. This module investigates β-lactamase, a bacterially produced enzyme that destroys β-lactam antibiotics

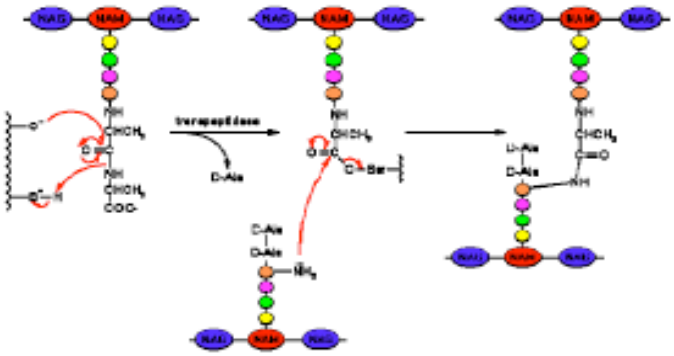
(penicillin, ampicillin, cephalothin for example). This module combines lecture material with laboratory experiments, computer simulations, and bioinformatics.

This material will utilize many virtual laboratory exercises as well as protocols and examples of "wet" labs. These laboratory exercises will provide the students with the opportunity to use equipment and techniques routinely used in molecular biology labs. Though this course is designed for the non-science student, exposure to this material is critical for the adult of the 21st century. They should have some basic understanding of the tools, procedures and data collection methods used as they are bombarded with genetic "news". The use of these protocols provides opportunities for hypothesis testing and data collection, therefore, there is potential for increasing the rigor of the protocols.

I. Overview: The Peptidoglycan, β-lactam antibiotics and β-lactamase

IA. The Peptidoglycan

Most bacteria have an outer layer, the peptidoglycan, that is responsible for preserving the shape of the bacteria and helps maintain the osmotic pressure. The peptidoglycan lies outside the cell. The building blocks are produced intracellularly and exported outside of the cell where the peptidoglycan is assembled. This structure is maintained by transglycosidases (connecting adjacent amino sugars) and transpeptidases (connecting the various layers). The transpeptidase enzymes, also called penicillin binding proteins, are responsible for this cross linking step (Fig. 1). There are many different transpeptidase enzymes studied. One common feature of these enzymes is that they contain a serine residue in their active site.



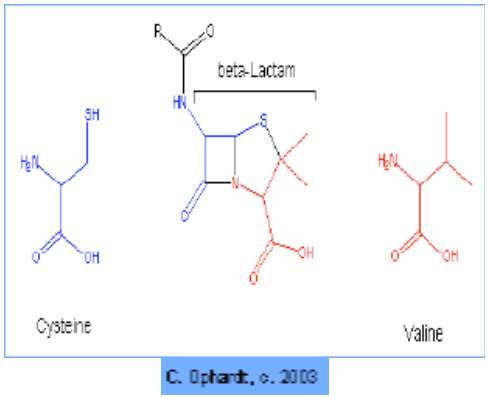
http://images.google.com/imgres?imgurl=http://www.chemsoc.org/exemplarchem/entries/2002/stanley/04_Site_of_Action/Site_8.jpg&imgrefurl=http://www.chemsoc.org/exemplarchem/entries/2002/stanley/04_site_of_action/site_of_action.htm&h=248&w=474&sz=24&hl=en&start=51&um=1&tbnid=Trze3OTrpkzTxM:&tbnh=67&tbnw=129&prev=/images%3Fq%3Dpeptidoglycan%26start%3D40%26ndsp%3D20%26svnum%3D10%26um%3D1%26hl%3Den%26rls%3Dcom.microsoft:en-us%26sa%3DN

Fig. 1. The transpeptidase reaction provides the cross links between different layers of the peptidoglycan

These cross links are pivotal for maintenance of the peptidoglycan. The transpeptidase enzymes catalyzes a bond between d-ala-d-ala residues of different layers (Fig. 1) If the cross link step is eliminated, the peptidoglycan is compromised, the cell swells and lyses. Antibiotics that interfere with this cross linking step are bacteriocidal.

IB. β-Lactam Antibiotics

β-lactam antibiotics represent a broad class of drugs that are effective due to their interference with peptidoglycan biosynthesis. This group of antibiotics is commonly used due to their low toxicity, ease of availability and is not very expensive. Within β-lactam antibiotics, there are two broad classes: the penicillin and cephalosporin classes. Both of these classes have the same active site and the same general structure– the β-lactam ring (Fig. 2). It is this structure, the



http://www.elmhurst.edu/~chm/vchembook/652penicillin.html

Fig. 2 The β-lactam bond common to penicillins and cephalosporins

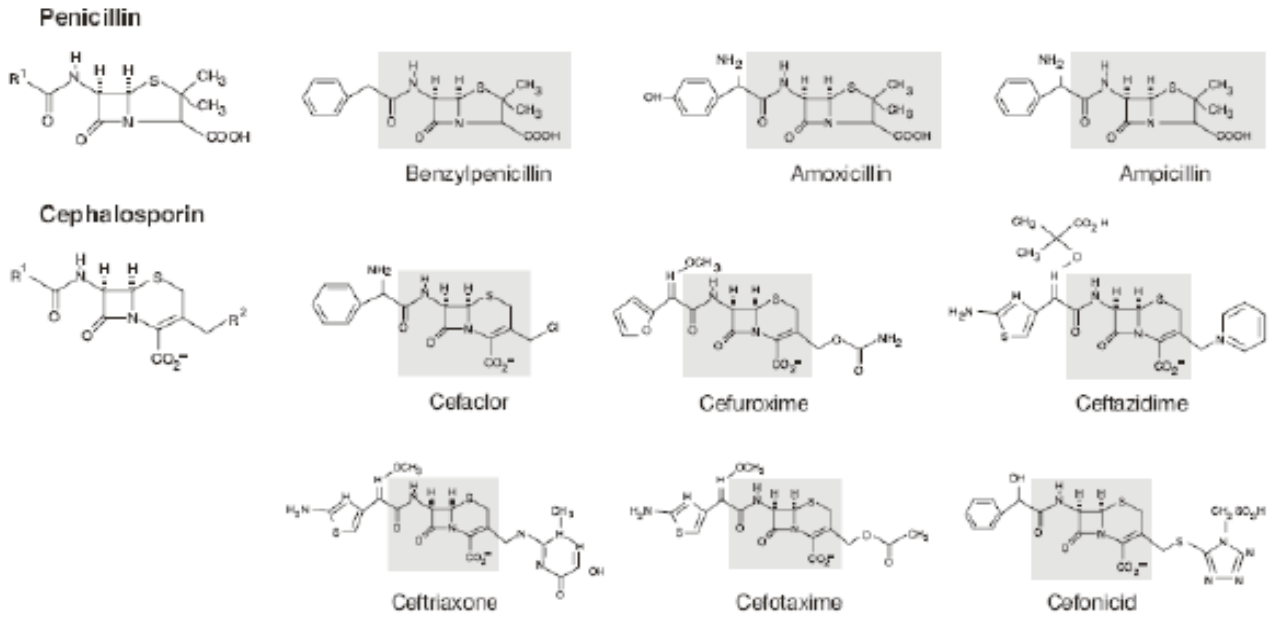
B-lactam ring, that is effective in killing bacteria. This bond competes with the d-ala-d-ala side chain that is used in the cross linking step in peptidoglycan biosynthesis. The transpeptidase enzyme that cross links the d-ala-d-ala binds to the antibiotic instead and is rendered ineffective, therefore, compromising the cross linking step in the structure.

The β-lactam drug, penicillin, was the first drug in this class to be used. Further developments led to the production of penicillin derived drugs that are classified based on the variety of bacteria they can successfully treat. If they are only effective for selected microbes, they are classified as narrow spectrum. As they become more diverse in their efficacy, the classification changes from moderate to broad spectrum. For example, the narrow spectrum β-lactam antibiotic (benzylpenicillin) is used to treat *Streptococci pyogenes* infections. Other pencillin derivatives include narrow spectrum penicillinase resistant (methicillin), moderate spectrum (ampicillin) followed by broad spectrum (amoxicillin with clavulanic acid) and extended spectrum (carbencillin) antibiotics.

There is a similar classification when discussing the cephalosporins. Here, they are divided into four generations of antibiotic development. The first two generations, represented by cephalothin and cefaclor respectively, are classified as moderate spectrum antibiotics. The third and fourth generation cephalosporins, represented by cefotaxime and cefepime, are classified as broad spectrum antibiotics.

The main difference between the penicillin and cephalosporin classes of antibiotics is the R groups (Fig. 3). The penicillin class tends to have one R group whereas the cephalosporin groups have two.

These R chains, as they become more complex, have been designed in an effort to combat bacterial resistance to these drugs.



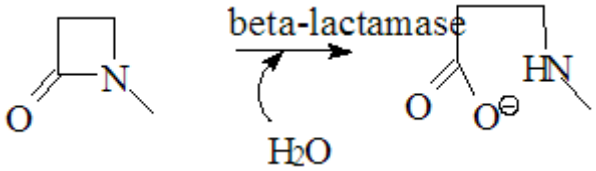
www.scielo.br/.../v82n5s0/en_v82n5s0a08f01.gif

Fig. 3. Representative β-lactam antibiotics

IC. Evolution of antibiotic resistance in bacteria: β-Lactamase

The generation of antibiotic resistance is a phenomenon exacerbated by overuse and misuse of antibiotics. However, therapeutic antibiotic use was not the underlying cause. The β-lactam antibiotics are manufactured by fungi growing in the soil. These organisms produce the antibiotics extracellularly effectively killing any susceptible microbe. In an effort to stay alive, microbes needed to evolve a mechanism to allow for survival. Though there are diverse β-lactam antibiotics, bacteria have been successful in developing resistance to this class of drugs. There are various mechanisms bacteria employ to resist β-lactam antibiotics but the focus of these experiments is bacterial production of β-lactamase. B-lactamase evolution and production provided the survival mechanism needed by the bacteria.

All β-lactamase enzymes share a common characteristic, a serine residue in the active site. This enzyme is effective by hydrolyzing the β-lactam bind rendering the antibiotic ineffective (Fig. 4)



www.hud.ac.uk/.../images/beta-lactamases01.gif

Fig. 4. β-lactamase hydrolyzes the β-lactam bond

Many diverse genera of bacteria are capable of producing β-lactamase enzymes. This is a very broad term that encompasses both chromosomally encoded enzymes as well as plasmid mediated enzymes. Using the same terminology as the β-lactam antibiotics, the enzymes are classified as narrow, broad and extended spectrum determined by the range of antibiotics they can hydrolyze. Interestingly, these enzymes have a serine residue in the active site similar to the transpeptidase enzymes that catalyze the cross linking reaction (and are the site for inactivation by β-lactam antibiotics).Due to their production many antibiotics cannot be prescribed.

ID. Conceptual Goals: The student should
1. Understand the function of the peptidoglycan
2. Be familiar with the biosynthesis of the peptidoglycan
3. Appreciate the efficacy of β-lactam antibiotics
4. Become aware of the health problems posed by the development of bacterially mediated β-lactam resistance
5. The role of β-lactamase

II. Bioinformatics as a Tool to Study β-lactamase

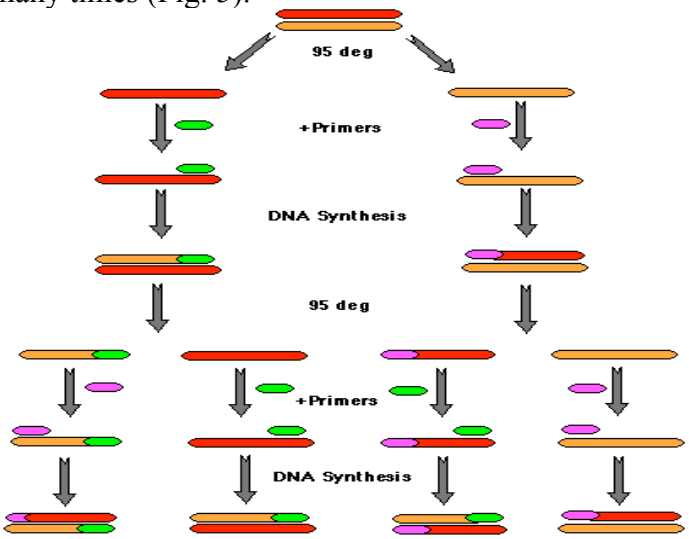
Bioinformatics is an extremely powerful tool used today as it allows computer simulations to investigate questions posed. Various tools include virtual molecular biology protocols and searching data bases to evaluate the putative function of a DNA sequence. Virtual web sites, using β-lactamase, can be used to illustrate many tools central to molecular biology. To examine the utility of these programs, this module uses pUR3 a plasmid engineered at the University of Richmond (UR). The pUR3 map and sequence are available to allow the type of virtual (and actual laboratories) that are included. Any other plasmid can be used for the virtual exercises as long as the sequence is available. The tools investigated will include the polymerase chain reaction allowing students to experience how primers are designed, selection of restriction enzymes to successfully cut out the β-lactamase gene, and agarose gel electrophoresis to demonstrate the results of restriction digestion. All of these procedures can be implemented as wet labs to enrich the virtual lab experience.

II.A.1: PCR: Selection of primers

Students have an opportunity to understand the design of the samples to facilitate successful amplification of the selected DNA. The requirements needed for this procedure include the sample of DNA, the dinucleotides as “building blocks”, appropriate buffer, the DNA polymerase and the target specific primers. While most of the reagents used are generic, the primers need to be designed specifically for the fragment of DNA.

The primers are essential for successful PCR as they identify the DNA region of interest for the DNA polymerase. Two primers are selected (both 5’ to 3’) – one for the forward strand and one for the reverse strand. Examination of the plasmid sequence identifies the coding sequence, in this case the β-lactamase gene. The primers chosen must flank this sequence while not being in the sequence.

It is also helpful if the primers are relatively close to the coding sequence desired. Using Primer 3 (http://frodo.wi.mit.edu/) and the entire plasmid sequence of pUR3, the students can experience the challenge (and sometimes difficulty) in obtaining the primers. As a result of successful PCR, the target site is amplified many times (Fig. 5).



http://members.aol.com/BearFlag45/Biology1A/LectureNotes/LNPics/Recomb/pcr.gif

Fig. 5 The Polymerase Chain Reaction

In this simulation the forward primer encroaches over a few base pairs on the forward strand. This simulation results in the students obtaining virtual data that indicates the size (in base pairs) of the successfully amplified product. In wet laboratories, this can be demonstrated by agarose gel electrophoresis.

To illustrate that PCR yields a greater quantity of a set piece of DNA and not a longer piece of DNA, various aliquots can be evaluated by agarose gel electrophoresis. An aliquot can be removed before any amplification, one after approximately 10 cycles and a sample at the conclusion of the process (Fig. 6).

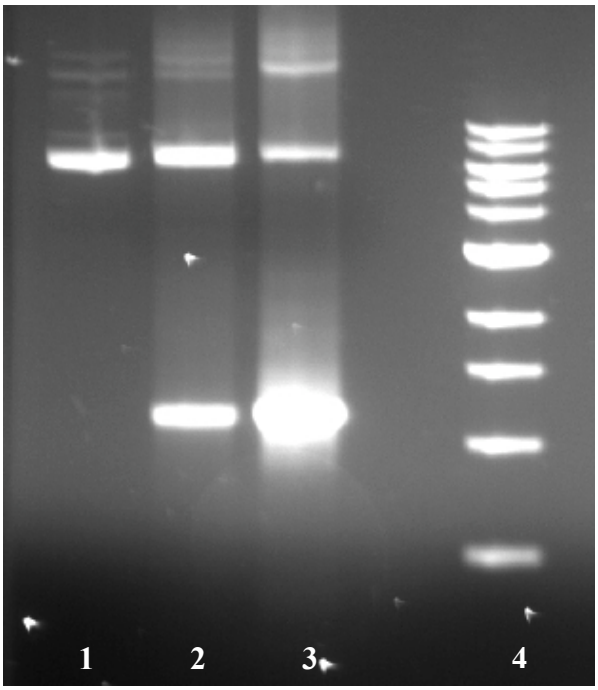
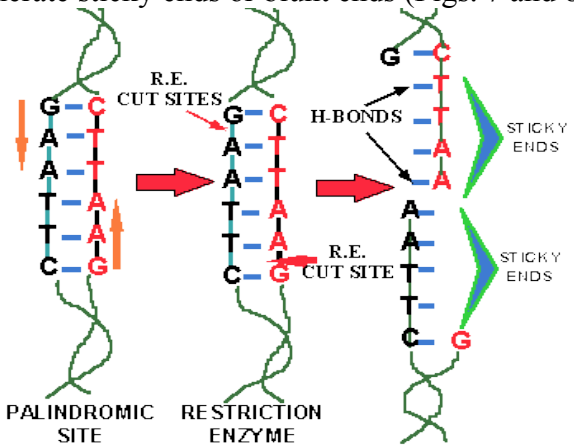


Fig. 6 Results of PCR. Lane 1 is the PCR mix prior to amplification, Lane 2 is after 10 cycles, Lane 3 is at the conclusion of the process and Lane 4 is the DNA marker.

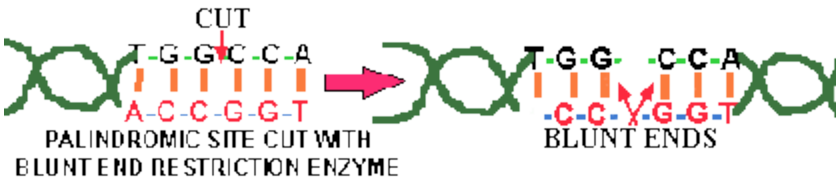
II.A.2. Use of NEB cutter to digest the plasmid

Restriction digestion is another powerful tool used routinely in molecular biology. This technique successfully "cuts out" the desired piece of DNA. Most bacteria use restriction enzymes as a means of protection from invading DNA by viruses. This process is specific and the enzyme only recognizes a set sequence of DNA base pairs called a palindrome. When the restriction enzymes "cut" the DNA, they can generate sticky ends or blunt ends (Figs. 7 and 8).



http://www.slic2.wsu.edu:82/hurlbert/micro101/images/mol10.gif

Fig. 7. Restriction digestion results in sticky ends



www.slic2.wsu.edu:82/.../images/mol10.gif

Fig. 8. Restriction digestion results in blunt ends

There are hundreds of restriction enzymes available for use when digesting DNA. As with designing primers for PCR, the restriction enzyme should cut the DNA outside the desired sequence allowing isolation of the target fragment of DNA (in this case, the β-lactamase gene) from the rest of the nucleic acid sequence.

The selection of the correct restriction enzyme can be investigated using a web-based site, NEB cutter (http://tools.neb.com/NEBcutter2/index.php) that shows a linear map of pUR3 with numerous restriction sites. Students, while referring to the plasmid map and sequence, will select different enzymes (or multiple enzymes) to successfully cut out the β-lactamase gene. Evaluation of the map (and sequence) allows estimation of the size of the gene (in base pairs). This can be confirmed by virtual gel electrophoresis as well as the actual restriction digestion. The virtual digestion (Fig.9 A) indicates a 1000 base pair fragment isolated from the rest of the plasmid and this is confirmed with the actual restriction digestion (Fig. 9 B).

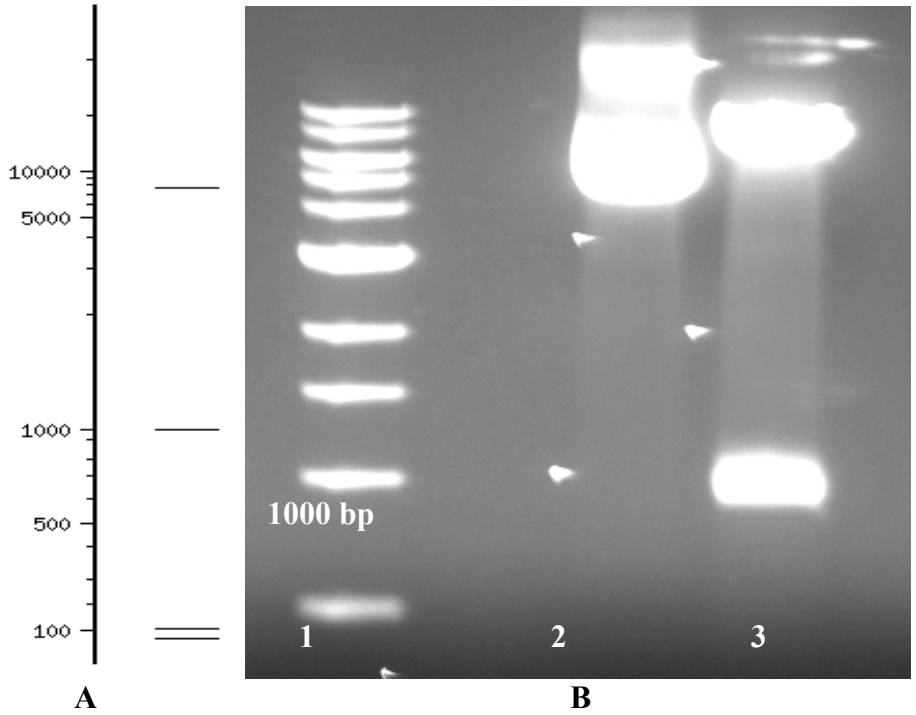


Fig. 9 A and B. Agarose gel electrophoresis: Restriction digestion of pUR3 with BspHI. A. Virtual digestion, B. Actual restriction digestion (Lane 1 is the DNA marker) in Lanes 2 (uncut control) and Lane 3 (cut)

III. Phylogenetic Analysis

The final component of this module allows the students to investigate the evolutionary relationship between the transpeptidase enzymes with the β-lactamase enzymes. Amino acid sequences of selected β-lactamase and transpeptidases are provided and the students can use a designated web site to investigate the possible role of each of these proteins. Once all amino acid sequences have been putatively identified, the SDS Workbench is used to construct a phylogenetic tree.

III.A. Putative function of amino acid sequences: Pfam

Students will investigate the putative function of the amino acid sequences provided. Both the transpeptidase enzymes and β-lactamase enzymes operate at the peptidoglycan, the transpeptidase enzymes successfully cross linking the d-ala-d-ala residues and the β-lactamase enzyme mimicking that structure inactivating the transpeptidase enzymes (penicillin family of antibiotics). Looking at the structure of the active sites of these enzymes reveals a conserved serine residue in both of these classes of enzymes. The next two exercises allow students to investigate the possible evolutionary relationship between the transpeptidase and the β-lactamase enzymes.

In preliminary investigations, students will be provided with seven amino acid sequences and will use a web based program Pfam (http://pfam.sanger.ac.uk/) to search for the putative function of these proteins in organisms. Students will be able to possible classify these sequences as either having transpeptidase or β-lactamase activity. This site also provides informative links for more in-depth analysis.

III.B. Construction of a Phylogenetic Tree: SDS Biology Workbench

Once the putative function of the amino acid sequences have been elucidated, the students will investigate if there is any evolutionary relationship between these proteins. To construct a phylogenetic tree, a web based program SDSC Biology Workbench (http://workbench.sdsc.edu.) will be uses. Importing the amino acid sequences into this program will result in the construction of the phylogenetic tree allowing students to decide whether there is evidence of an evolutionary relationship between these two enzymes.

III.C. Bioinformatics and Phylogenetics Goals: Students will understand

1. How PCR works, how primers are selected and the role of all the components
2. The selection of an appropriate restriction enzyme(s) to cut out the desired sequence
3. Bioinformatics as a powerful tool in molecular biology
4. Determination of the putative role of amino acid sequences
5. Investigation of the evolutionary relationship between protein sequences

IV. Laboratory Protocols to Accompany the Simulations

A. Tools of Molecular Biology: introduces the students to micropipettors, and sample preparation.
B. PCR for the β-lactamase gene
C. Plasmid isolation of pUR 3 from *E. coli*
D. Restriction digestion of PCR products and isolated pUR3
E. Agarose gel electrophoresis

Appendix

I. On-line sources

Cell wall of bacteria:
http://www.cehs.siu.edu/fix/medmicro/pix/walls.gif
http://www.bact.wisc.edu/Microtextbook/index.php?module=Book&func=displayarticle&art_id=89&theme=Printer

Peptidoglycan biosynthesis:
http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/ppgsynanim.html
http://images.google.com/imgres?imgurl=
http://www.chemsoc.org/exemplarchem/entries/2002/stanley/04_Site_of_Action/Site_8.jpg&imgrefurl=http://www.chemsoc.org/exemplarchem/entries/2002/stanley/04_site_of_action/site_of_action.htm&h=248&w=474&sz=24&hl=en&start=51&um=1&tbnid=Trze3OTrpkzTxM:&tbnh=67&tbnw=129&prev=/images%3Fq%3Dpeptidoglycan%26start%3D40%26ndsp%3D20%26svnum%3D10%26um%3D1%26hl%3Den%26rls%3Dcom.microsoft:en-us%26sa%3DN

β-lactam antibiotics
http://www.elmhurst.edu/~chm/vchembook/652penicillin.html
www.scielo.br/.../v82n5s0/en_v82n5s0a08f01.gif

β-lactamase activity
www.hud.ac.uk/.../images/beta-lactamases01.gif

PCR:
http://members.aol.com/BearFlag45/Biology1A/LectureNotes/LNPics/Recomb/pcr.gif
http://www.dnalc.org/ddnalc/resources/pcr.html animation
http://learn.genetics.utah.edu/units/biotech/pcr/ animation

Restriction digestion:
http://www.slic2.wsu.edu:82/hurlbert/micro101/images/mol10.gif
http://www.wiley.com/legacy/college/boyer/0470003790/animations/agarose/agarose.htm animation
http://www.slic2.wsu.edu:82/hurlbert/micro101/images/LigaseAnimation6.gif animation
http://www.dnalc.org/ddnalc/resources/restriction.html animation

2. References

Peptidoglycan biosynthesis
http://www.textbookofbacteriology.net/structure.html

Bush, K. 1988. Recent Developments in β-Lactamase Research and Their Implications for the Future. Reviews of Infectious Diseases, Vol. 10, No.4: 681 – 690.

Medeiros, A. 1997 Evolution and Dissemination of β-Lactamases Accelerated by Generations of β-Lactam Antibiotics. Clinical Infectious Diseases, Vol. 24, S1: S19-S45.

Meroueh, S. O, Minasov, G, Lee, W., Shoichet, B., and S. Mobashery. 2003 Structural aspects for evolution of beta-lactamases from penicillin-binding proteins. Journal of the American Chemical Society, Vol. 125 (32): 9612-9618.

Neu, H. 1992. The Crisis in Antibiotic Resistance. Science, New Series, Vol. 257, No. 5073: 1064 – 1073.

3. Materials for the protocols

A. Luria Bertani (LB): Broth and / or agar plates

10 g Tryptone
5 g Yeast Extract
10 g Sodium chloride
14 g agar
1 L dH_2O
Autoclave

B. Ampicllin: final concentration 50µg/ml

C. pUR3: available on request and can be sent on an LB – Ampicillin plate

D. Primer design

1. Forward and reverse primers: available using Primer 3 (http://frodo.wi.mit.edu/)

LEFT PRIMER: TTTGCCTTCCTGTTTTTGCT
RIGHT PRIMER: ATAATACCGCGCCACATAGC

E. PCR Reaction: PCR beads Amersham PureTaq ready to go PCR beads---#27-9558-01

F. PCR Thermocycler:

1	95 ^{o} C		5 min
2	95 ^{o} C		30 sec
3	55 ^{o} C		30 sec
4	72 ^{o} C		1.2 min
	2-4	29 X	
5	72 ^{o} C		10 min
6	4 ^{o} C		for ever

H. Plasmid isolation protocol: Qiagen kit 27104 plasmid min-prep kit for 50 plasmid preparations

I. Restriction digestion: BspHI, RO517S, New England Biolabs

J. Bovine Serum albumin: 200 µg/ml

K. Agarose: prepare 0.8% gels in TBE

L. TBE:

TBE buffer (electrophoresis) 1liter – 5X
54 g Tris base
27.5 g Boric acid
20 ml 0.5M EDTA

M. DNA marker: Quick-Load™ 1 kb DNA Ladder New England Biolabs

N. Loading dye: Blue-Orange 6X loading dye, G1881, Promega

O. Ethidium bromide: 10 mg/ ml for stock, add 1µl/10 ml agarose