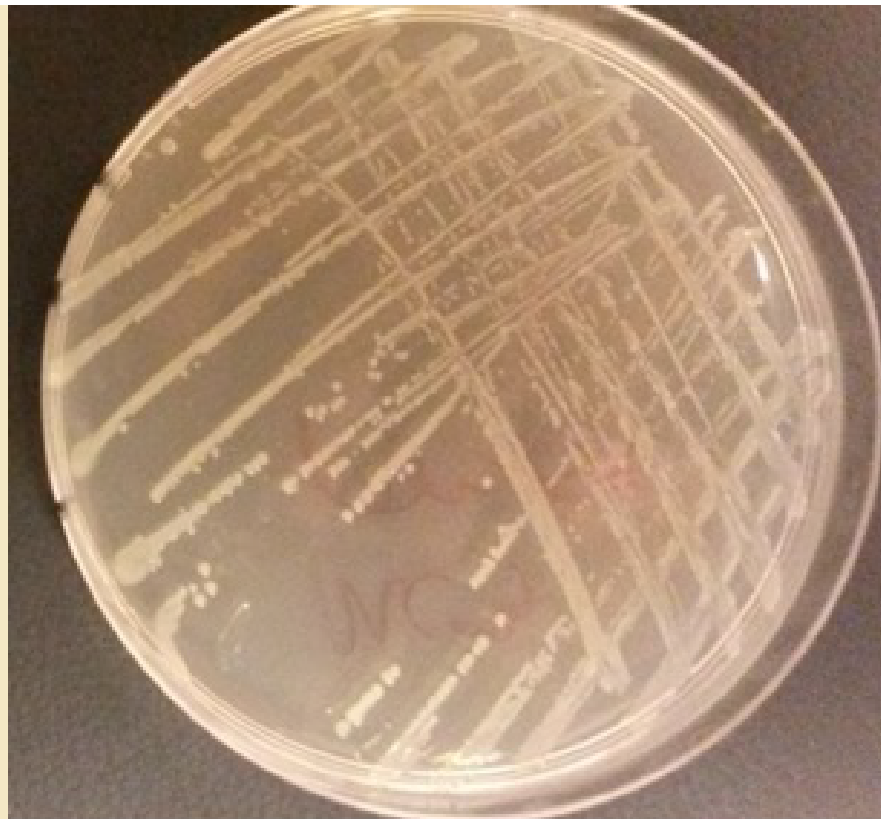Master Thesis

Elisabeth Eika

Mecillinam resistance in clinical isolates of *Escherichia coli*

Telemark University College

Faculty of Arts and Sciences

Master of Environmental Health

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Abstract

Resistance to the β-lactam mecillinam was observed in 11 clinical isolates of *Escherichia coli* (MC-isolates) from urinary tract infections. How this resistance arose, is not known.

The clinical isolates differ from normal E. *coli* by showing abnormal cell shape and slow growth. Formation of the normal *E. coli* rod shape involves two gene clusters, *mrd* and *mre* located respectively at 14 min and 71 min on the *E. coli* chromosomal map, *mrd/mre* mutants may show mecillinam resistance.

This study attempt to test whether the MC isolates are *mrd/mre* mutants by introducing plasmids carrying *mrd/mre* by electroporation and looking for restoration of normal morphology and mecillinam sensitivity. Attempts to introduce a plasmid carrying the *mrd* genes were only successful for one of the eleven MC isolates. The plasmid did not restore the normal phenotype – i.e. the strain kept its mecillinam resistance and coccoid morphology.

Because of the lack of results, different approaches to introduce desired genes have been theoretically explored.

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Glossary

Ara operon:	Operon containing genes coding for arabinose catabolism under the control of the AraC repressor and the cAMP repressor protein
β - lactam:	Broad class of antibiotics that contain a β-lactam ring in their molecular structure
IPTG :	Gratuitous inducer of the lac operon in *E. coli*
Electroporation :	Method for transforming bacteria using electric shock
Exogenous DNA:	Genetic material that originates outside of the organism of concern
F-prime plasmids:	A derivative of the conjugative F plasmid containing a segment of chromosomal DNA
Lac operon:	Operon containing genes coding for lactose catabolism under the control of the LacI repressor and cAMP receptor protein
λ-transducing phage:	A derivative of bacteriophage lambda containing chromosomal genes. Specialized transducing phage. A restricted set of bacterial genes is transferred into the bacteria.
Mecillinam:	β - lactam antibiotic, specifically binding to PBP2. Also known as amdinocillin
MIC:	Minimal Inhibitory Concentration
MC-isolates :	Mecillinam resistant clinical isolates used in this study
***mrd* region**:	Gene cluster responsible for cell shape located at 14 min on the *E.coli* chromosomal map
***mre* region** :	gene cluster responsible for cell shape located at 71 min on the *E. coli* chromosomal map
PBP:	Penicillin Binding Proteins, characterized for their affinity for and binding of penicillin. These proteins are involved in the final stages of synthesis of peptidoglycan
PBP2:	Penicillin binding protein 2. The point of attack for mecillinam
Peptidoglycan:	Important compound found in the bacterial cell wall. Also known as murein
Plasmid:	Small circular DNA molecule that can replicate independently of chromosomal DNA
pTB59:	Plasmid construct carrying *mrd* genes under the control of the lac operon and ampicillin resistance gene

pLP31: Plasmid construct carrying *mre* genes under the control of the ara operon and chloramphenicol resistance gene

Preface

Present text is written as a master thesis at Telemark University College, department of environmental and health sciences, and represents 60 credits.

All laboratory work has been performed at HiT's laboratories using HiT equipment.

My supervisor has been professor Andrew Jenkins, to whom I would like to direct my appreciation and thanks for guiding me down the right path, helping me find inspiration and passion, and off course sharing his enormous knowledge about the microbiological world.

We've had some great fun this year!

I would also like to thank lab-technician Karin Brekke Li, always being positive, helpful and patient when needed, the very helpful librarians at HiT, assisting me to search for relevant literature, and Dr. Med. Nils Grude for sharing his knowledge.

All clinical *E. coli* isolates were provided by professor Andrew Jenkins and Dr. Med. Nils Grude.

Bø, mai 2014

Elisabeth Eika

Thesis - the aim of this study

Clinical MC isolates show abnormal cell shape and resistance towards the β-lactam antibiotic mecillinam. Initial hypothesis is that the mecillinam resistance is caused by genetic mutations in terms of the *mre* or *mrd* gene clusters, which are responsible for several processes involved in the formation of the bacterial cell wall.

This study investigates if introduction of properly functional genes will restore normal cell shape and sensitivity towards mecillinam.

Introduction

The bacterial cell wall

Bacteria come in several shapes and many sizes. Most bacteria range from 0,2 - 2,0 μm, and they have a few basic shapes; coccus, rod-shaped bacillus and spirilla and spirochetes (Figure 1). They can also be filamentous, irregular and vibrios amongst others. The cell wall of the bacterial cell is a complex, semigrid structure, responsible for the cell shape. Not only does it decide the shape of the cell, but it also surrounds the underlying fragile plasma membrane, and protects not only the membrane, but also the interior of the cell from adverse changes in the outside environment (Tortora, Funke et al. 2007). The essential cell wall polymer of most bacteria is called peptidoglycan, or murein. Murein is a heteropolymer consisting of glycan strands which are crosslinked by peptides (Ghuysen and Hackenbeck 1994). This makes the cell wall strong;

Figure 1: Different shapes in bacteria. Photo: http://faculty.cbu.ca/cglogowski/images/prokaryoteshapes.jpg

it is held together by covalent bonds with strength similar to the bonds holding the atoms of a diamond together. The cell wall is also called the bacteria's exoskeleton or sacculus (Koch 2000). The

peptidoglycan structure consists of a repeating disaccharide attached by polypeptides to form the "mesh" that surrounds and protects the entire cell. The disaccharide portion is made up of monosaccharides called N-acetylmuramic adid (NAM) and N-acetylglucosamine (NAG), both are which related to the more familiar monosaccharide glucose. Figure 2 shows this structure.

Figure 2: NAM - NAG structure with peptide bridges. Photo: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/N/NAG_NAM.gif

Alternating NAG and NAM molecules are linked in rows forming a carbohydrate "backbone" 10-65 sugars long. Adjacent rows are linked by polypeptides consisting of four amino acids attached to the NAM's in the backbone. (Peptide bridge) The structure of the polypeptide link can vary, and the
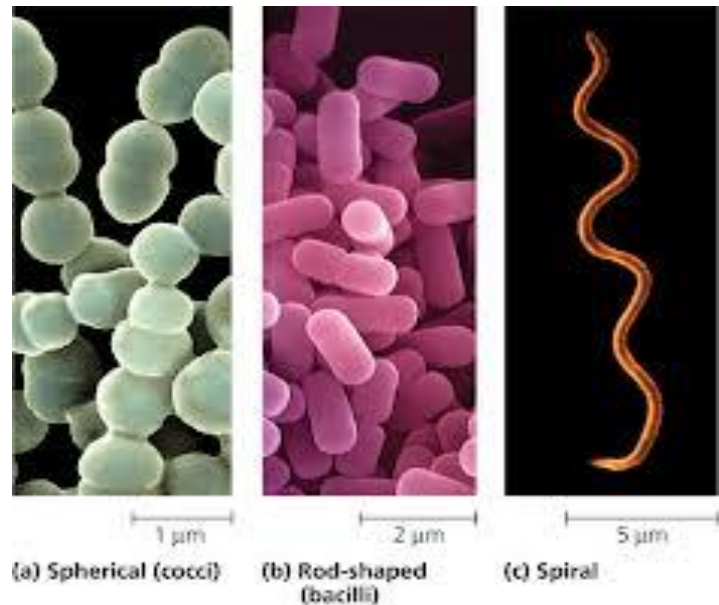
amino acids occur in an alternating pattern of D and L forms. This is unique for the bacterial cell wall; the amino acids found in proteins are all L forms. Parallel tetrapeptide side chains may be directly linked or bonded to each other by a peptide cross-bridge (a short chain of amino acids) (Tortora, Funke et al. 2007).

Gram-negative bacteria

Gram-negative bacteria have a thin cell wall, only consisting of one or a very few layers of peptidoglycan and an outer membrane. Figure 3 clearly shows the difference between gram positive and gram negative bacteria relative to the cell wall.

The peptidoglycan is bonded to lipids covalently linked to proteins, called lipoproteins, in the outer membrane and is located in the periplasm (a gel-like fluid between the outer membrane and the plasma membrane). The periplasm contains a large amount of transport proteins and degradative enzymes. In the outer membrane of a Gram-negative cell, we find lipopolysaccharides (LPS) lipoproteins and phospholipids, and the membrane has several specialized functions.
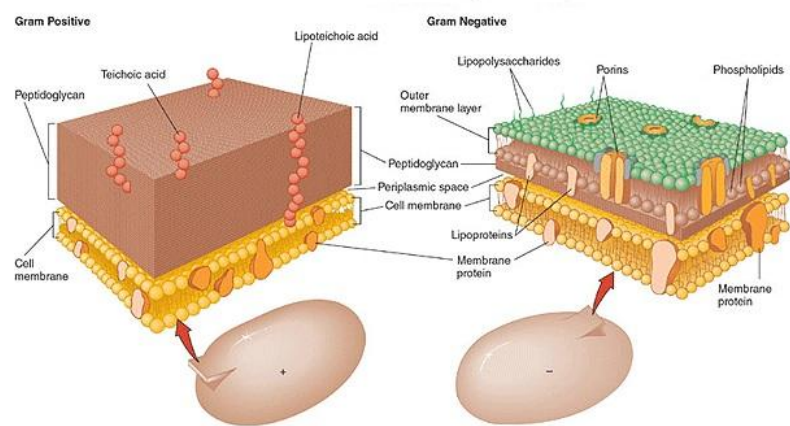
Figure 3: The differences in cell wall structure between Gram-negative and Gram-positive bacteria. Photo:
http://water.me.vccs.edu/courses/env108/clipart/cellwall.gif

The polysaccharide portion of the LPS is composed of sugars called O-polysaccharides; these function as antigens and are useful for distinguishing species of Gram-negative bacteria (Ghuysen and Hackenbeck 1994).

Cell cycle

A cell reproduces by duplicating its contents in an orderly sequence of events, and then dividing in to two new cells. This is known as the cell cycle. In rod shaped bacteria, such as *E. coli*, the cell elongates until the cell is approximately twice its original length, and then it divides into two new cells – this process is called binary fission. A partition called the septum forms at the middle of the elongated cell, and here inward growth of the cytoplasmic membrane and cell wall proceeds until the two daughter cells are pinched off (Tortora, Funke et al. 2007). When a newly divided cell has doubled its size and divides, a generation has occurred. Usually all cellular constituents increase

proportionally during the growth cycle (There are exceptions). *E. coli* can complete the cell cycle in about 20 minutes under the best nutritional conditions.

For cell division to occur, several proteins called Fts proteins are essential. A key protein in the Fts group is the FtsZ protein, which is well studied in *E. coli*. The Fts proteins interact and form a division apparatus – the divisome, and in *E. coli* the formation begins with the attachment of molecules of FtsZ in a ring around the cell cylinder at the precise midpoint of the cell (Madigan, Martinko et al. 2006). The divisome also contains Fts proteins like FtsI that are needed for peptidoglycan synthesis. FtsI is a penicillin-binding protein, one of several present in the cell, which are so called because their activities are inhibited by the antibiotic penicillin.

Figure 4: A simplified form of cell division in *E.coli*. Photo: http://www2.warwick.ac.uk/fac/sci/chemistry/research/arodger/arodgergroup/people/msrgbg/conferences/a_tptical_cell_cycle_in_e._coli_s.png

The localization of the FtsZ-ring, the cell midpoint, is facilitated by a series of proteins called Min proteins. The Min proteins ensure that the divisome forms only at the cell center, and not at the cell poles.

Another Fts protein called FtsK, along with several other proteins, assists in the process where the two copies of the chromosome are pulled apart, each to its own daughter cell. This happens while the cell is still elongating. As constriction occurs, the FtsZ-ring starts to depolymerize, and this triggers the inward growth of wall material, that in the end seals off the two daughter cells. Bacterial cell division cannot occur without functional Fts proteins (Madigan, Martinko et al. 2006, Tortora, Funke et al. 2007). Figure 4 shows a simplified schematic of cell division.

Morphology

We have now seen that specific proteins direct the cell division in prokaryotes; other specific proteins define cell shape. In prokaryotes the major shape-determining protein is called MreB. This protein forms a simple cytoskeleton, and presumably this cytoskeleton defines the cell shape by recruiting other proteins that orchestrate cell wall growth in a specific pattern (Tortora, Funke et al. 2007). After deletion of the *mreB* gene in *E. coli*, the cell shape changed, becoming round or irregular. The rod shape in *E. coli* also depends on the genes *envB*, *mreC* and *mreD*, located in the *mre* cluster together with *mreB* (Wachi, Doi et al. 1987) (Cabeen and Jacobs-Wagner 2005). In the *mrd* (Murein D) operon, the *rodY, mrdA* (*pbpA*) and *mrdB* (*rodA*) genes are found, and these encode MrdA (PBP2) and MrdB (RodA), which participate in the maintaince of the rod shape. The *mre* cluster can be found at 71 min on the chromosomal E. coli map, whileas *mrd* is located at 14 min (Fig. 5). Mutants

isolates of *E. coli* which shows a spherical cell-shape instead of the typical rod-shape showed mutations located either in the mre or the mrd region of the gene map (Wachi, Doi et al. 1987). PBP2 and PBP3 (FtsI) are unique counterparts amongst the PBP's in *E. coli*. PBP2 is specifically required for cylindrical murein synthesis during cell elongation, but dispensable for septal murein synthesis during cell constriction while PBP3 is specially required for septal murein synthesis but dispensable for cylindrical murein synthesis. In the family of polytopic membrane proteins we find RodA and the division protein FtsW, and evidence suggests that these proteins are required for proper function of PBP2 and PBP3 (Wachi, Doi et al. 1987) (Bendezu and de Boer 2008).
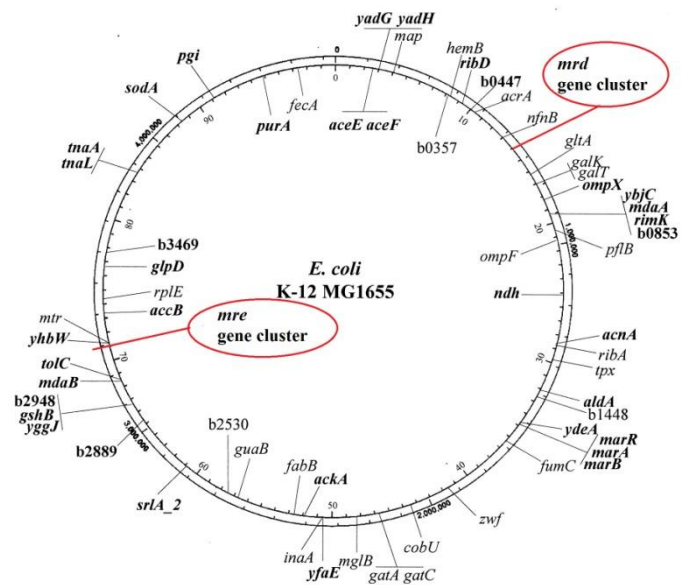

Figure 5: Chromosomal map of E.coli K12 strain showing location of mre and mrd gene clusters.
Photo:http://jb.asm.org/content/182/12/3467/F2.large.jpg

Antibiotics and antibiotic resistance

Antibiotics are low-molecular weight substances usually produced by microorganisms, and they are able to enter bacteria and kill them (bactericidal) or inhibit their growth (bacteriostatic). Antibiotics can be classified according to their chemical structure and their diverse action mechanisms. Antibiotics can target components of the cell wall, components of the cytoplasmic membrane or the biosynthetic apparatus of the bacteria.

When exposed to antibiotic, the bacteria sometimes develop mechanisms that makes the antibiotic ineffective – resistance mechanisms. Like the antibiotics themselves, these mechanisms can also be quite diverse and may be specific or unspecific. Some bacteria already have naturally (primary resistance) mechanisms present, but acquired (secondary) resistance mechanisms play an important role. For transmission and formation of the latter, plasmids are often essential (Seltmann and Holst 2001).

Plasmids

Some plasmids play an important role in the spread of antibiotic resistance among bacteria. Many bacteria possess plasmids, which are small circular DNA molecules. They can be present in many or one/a few copies per cell. Generally, plasmids carry genes that are not essential to basic bacterial function, but they may play an important role in in the growth or life cycle of their bacterial host in special environments. Plasmids possess their own origin of replication, and can therefore replicate independently of the bacterial chromosome. Plasmids that are related to each other are often

incompatible, in the sense that when two related plasmids are present only one of them persists in the cell (Alberts 2008).

Mutations

Bacteria constantly undergo mutations. This may lead to changes in properties, such as resistance to antibiotics.

There are several types of mutations, but one type of mutation consisting of insertions or deletions change the primary sequence of the encoded polypeptide typically in a major way, and such changes inevitably result in complete loss of gene function (Madigan, Martinko et al. 2006).

Mutations may be identified by the use of DNA sequencing.

Another way to detect mutations is to introduce a second, intact copy of the mutated gene. This is known as a complementation test (Pierce, 2002).

Escherichia coli

E. coli is a gram-negative, facultative anaerobe rod-shaped bacteria (Fig. 6). The generation time for *E. coli* in the intestinal tract is estimated to be 12-24 hours, while in the laboratory it is as little as 15-20 minutes. A large number of different strains belonging to this species have been isolated and characterized. Most strains have their normal habitat in gut of man and animals, some possess virulence factors that enable them to cause infections at other sites,

Figure 6: Escherichia Coli. Photo:
http://www.electronicproducts.com/uploa

such as the urinary tract or the intestinal tract itself (Mims 2004), *E.coli* is the most common cause of urinary tract infections (Grude 2006).

E. coli usually spreads by contact and ingestion (fecal – oral route), it may be food associated or from environmental sources. It possesses somatic, flagellar, capsular and fimbrial antigens, and this can be used to characterize strains by serotyping. Pathogenic *E. coli* strains can cause urinary tract infections, diarrheal diseases, neonatal meningitis and septicemia. A variety of virulence factors have been identified;

- Endotoxin: present in all strains.
- Adhesins: fimbriae associated with urinary tract infection and colonization factors associated with gastrointestinal tract infection in both humans and animals.
- Capsule is present in some strains, and this may be associated with adhesins.
- Enterotoxins associated with diarrheal disease.

There are a wide range of antibacterial agents available for treatment, but there are incidences of resistance. The resistance towards the antibacterial agents is often plasmid-mediated (Mims 2004).

Mecillinam

Mecillinam is a narrow spectrum antibiotic (which is often preferred as treatment to avoid development of resistance) and has been used successfully for treating urinary tract infections (UTI) and also typhoid and paratyphoid fever (Tybring and Melchior 1975, Clarke, Geddes et al. 1976, Leo Pharma 2008). It is only considered to be active against gram-negative bacteria, such as *E. coli* which it is considered to be highly active against (Neu 1985). Mecillinam cannot be absorbed from the gastrointestinal tract, and therefore needs to be administered parenterally. For oral treatment pivmecillinam; an ester of mecillinam, can be administered. Pivmecillinam is enzymatically hydrolysed to mecillinam after absorbtion. Resistance against mecillinam has been reported to be very low, ever since its introduction in clinical use during the 1970's (Tybring and Melchior 1975, Clarke, Geddes et al. 1976, Leo Pharma 2008).

From year 2000 – 2006 the number of resistant strains of *E. coli* has maintained stable. About 80% of *E. coli* present in UTI's is fully suspectible to mecillinam (Leo Pharma, 2008).

Mecillinam (formerly called FL1060) is a β-lactam closely related to the penicillins, but its precise chemical structure and mode of action are different (Fig. 7).

β-lactam antibiotics are recognized because they have a core structure - a β-lactam ring- that is similar in all members of the family. Almost all of the β-lactams derive their antimicrobial activity by inhibiting bacterial cell wall synthesis. Mecillinam specifically binds to penicillin binding

Figure 7: chemical structure of mecillinam. Photo: Leo Pharma AS

protein 2 (PBP2) while other β-lactams preferentially bind to PBP's 1 and 3. PBP2 is a murein-elongation initiazing enzyme, and antibiotics binding to PBP2 seems to produce round cells (Neu 1983).

The lytic effect of mecillinam is dependent on the time growing cells are exposed to the compound. The growing cells needs to be under the influence of the substance for relatively long periods of time for the mecillinam to exhibit its bactericidal effects. In contrast to penicillins, mecillinam does not cause cuts in the bacterial envelope, but a weakening of the sacculus seems evident (Neu, 1985). When exposed to mecillinam *E. coli* seems to lose the normal distinct separation between the phase
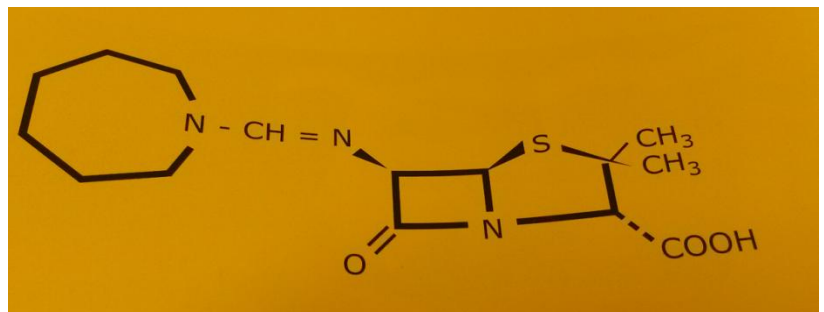
of cell elongation and the phase of cross wall formation, causing cells to become short and swollen. An unbalanced separation like this is followed by incomplete segregation of the cell contents and loss of the normal parallel-orientated division.

The mechanism of resistance in clinical strains is unknown, but mecillinam resistant isolates selected in lab experiments are mutants in certain *mre* and *mrd* genes. These mutants have coccoid morphology (Bendezu and de Boer 2008).

Expression vectors used in this study

The *lac* operon

In *E. coli* the *lac* operon controls uptake and catabolism of the disaccharide lactose, one of the major carbohydrates found in milk. Two regulatory proteins, CAP and LacI enables *E. coli* to use lactose when glucose is absent. LacI binds to the *lac* operator *lac*O and prevents transcription in the absence of lactose. When lactose is present LacI is released from *lac*O and the block on the transcription is removed. The same effect may be acchieved using the gratious inducer IPTG (a molecular mimic of lactose). High level expression of the *lac* operon requires binding of the CAP protein to the CAP site.

Figure 8: The lac operon. Shows how the absence or presence of lactose regulates gene expression. Photo: http://upload.wikimedia.org/wikipedia/commons/d/d2/Lac_operon-2010-21-01.png

High levels of glucose prevent CAP binding. The CAP protein aids the binding of RNA polymerase, and *lac* genes are transcribed. The control region of the *lac* operon can respond to and integrate two different signals so that the operon is only highly expressed when two conditions are met; glucose must be absent and lactose must be present. If these conditions are not met, the *lac* operon will remain inactive (fig.8)(Pierce 2002, Alberts 2008). In this study the *lac* operon is used as a genetic switch in the plasmid pTB59 (Fig 36).

The arabinose operon

Another example of a combination of positive and negative gene control is the *ara* operon present in *E. coli*. The *ara* operon consists of three structural genes, *ara*A, *ara*B and *ara*D, that code for the catabolic enzymes kinase, isomerase and epimerase, and a C-gene that produces the AraC protein
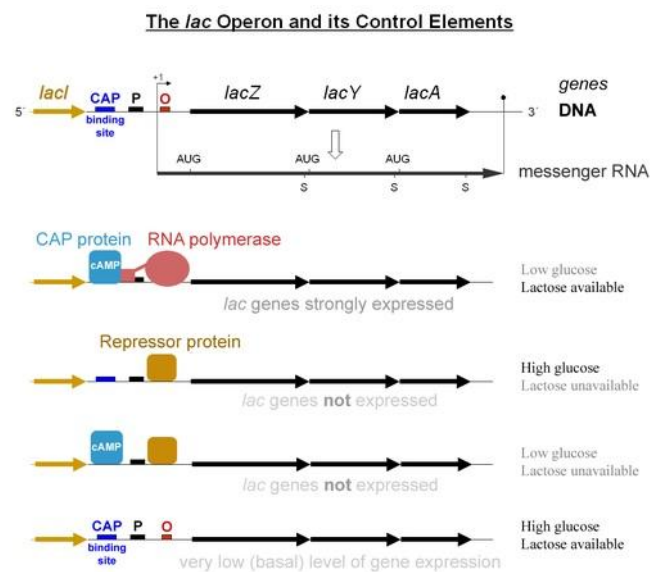
that together with arabinose turns on the *ara* operon. Continuous research on the *ara* operon discovered a phenomenon called DNA-looping, now known to be widely used in gene regulation. The arabinose system enables E. coli to take up the pentose L-arabinose and use as an alternative carbon source. In presence of arabinose the AraC protein stimulates initiation of mRNA synthesis from the promoters. The AraC protein acts both positively by stimulating transcription in the presence of arabinose and also negatively in the absence of arabinose by repressing transcription initiation. When arabinose is not present, the AraC protein binds to two sites in the *ara* operon, causing the operon to form a DNA-loop that blocks
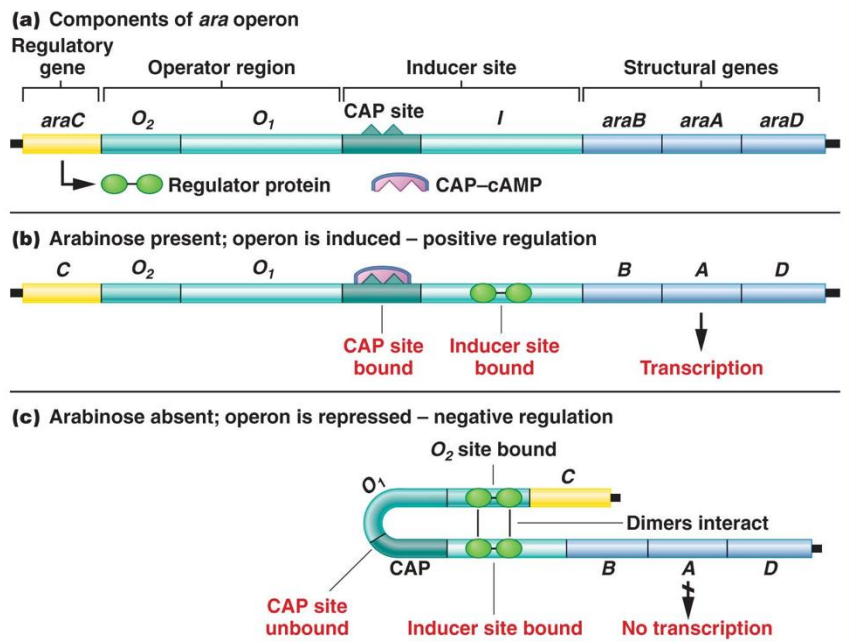

Figure 9: Shows the ara operon. a) shows the components of the ara operon. b) describes how the presence of arabinose stimulates the transcription of the araA, araB and araD genes. The AraCprotein binds to the araI region and the CAP-cAMP complex binds to a site adjacent to araI. C) shows hos the AraC protein binds to both the araI and araO regions when arabinose is absent, preventing transcription. Photo: http://classconnection.s3.amazonaws.com/883/flashcards/443883/png/picture_81334535655395.png

access of RNA-polymerase, and mRNA is not transcribed. This is shown in figure 9 (Schleif 2000).

Transferring foreign DNA into bacteria

There are three processes by which exogenous DNA may be introduced into a bacterial cell; transformation, conjugation and transduction.

This study has used a variant of transformation called electroporation, but only with limited success, and this lead to the discussion of possible benefits by the use of other transfer methods .

Electroporation

Transformation of bacteria is the direct uptake and incorporation of exogenous DNA from the surroundings through the cell membrane(s) . One way of achieving transformation is called electroporation. Electroporation is a technique now widely used in laboratories around the world as a method for introducing foreign genes into cells. In this study we chose electroporation as method because the standard cold-calcium chloride method (Maniatis et.al 1982) previously has failed in similar attempts.

This is being done by exposing the cell to a sufficiently large electric field. When exposed, the plasma membrane of the cell is changed, so that molecules can pass through.

The electroporation causes "electropermeabilization" of the membrane; a dramatic increase of permeability (or conductivity) of the cell. Pores in the membrane are formed because of the electric field, and here molecules can pass through and into the cell. (Weaver and Chizmadzhev 1996, Miklavcic, Mir et al. 2010)

Conjugation and F-prime plasmids

Direct transfer of genes from one bacterial cell to another is called conjugation. This is mediated by conjugative plasmids that transfer genetic material between cells by direct cell to cell contact or by a bridge-like connection (pilus) between two cells (Figure 10). The donor cell transfers genetic material (most often plasmid, but it can also be a transposon) to the recipient cell. Conjugation is a highly efficient method for DNA transfer as cells in a culture with donor cells will rapidly acquire donor properties and then act as donor cells themselves. When conjugation occurs only a single strand of DNA is transferred, and this should prevent the DNA from being cut by restriction enzymes. F-prime plasmids are derivatives of the conjugative plasmid F, which have a piece of the bacterial

Figure 10: Conjugation between an F$^+$ cell (to the left) And an F$^-$ cell (to the right) resulting in the F$^-$ cell becoming an F$^+$ cell. Photo: http://www.personal.psu.edu/czc5161/blogs/testing/conjugation.jpg

chromosome incorporated (Pierce 2002). F plasmids have a high frequency transfer rate. In my experiment, if the problem is to get the plasmid incorporated in the bacteria, the use of F-prime plasmids containing the desired *mre* or *mrd* genes could be useful, as this is an extremely efficient method for plasmid transfer. But, if my cells are F$^+$, the conjugation will not take place. Suitable F-prime factors for *E. coli* are described (Low 1972).
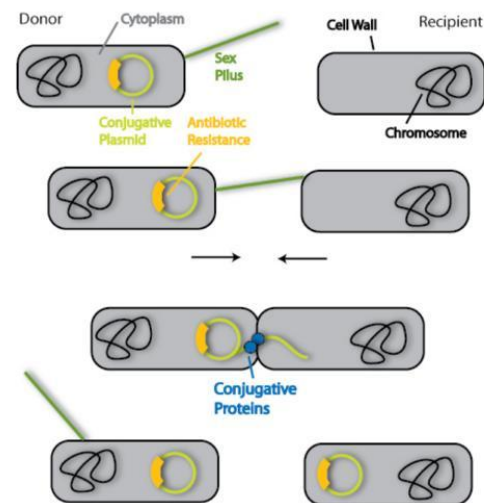
λ-transducing phages

Gene transfer by bacteriophages is called transduction

Phages such as λ-transducing phages, offer a number of advantages as gene transfer vectors. One of the biggest advantages using phages is that they have a high efficiency of transferring DNA into bacterial cells. Another advantage is that about a third of the λ genome is not essential for reproduction and infection. This third-part of the genome (about 15 kb) can be replaced with as

much as 23 kb of foreign DNA, and the λ phage will still inject its DNA into the bacterial cell and reproduce (Pierce 2002). The λ phage contains an enzyme called lambda integrase, and this enzyme mediates the covalent joining of the viral DNA to the bacterial chromosome. Now the viral DNA is a part of the bacterial chromosome and is replicated passively as a part of the host's DNA (Alberts 2008).

Barriers to plasmid transfer

In naturally-occuring bacterial strains there are often barriers to DNA transfer

Bacteria may contain mechanisms that destroy the incoming DNA, or prevent its establishment.

Examples of such mechanisms are restriction modification or plasmid incompability.

Restriction-modification systems

Different strains of bacteria make different restriction nucleases. Restriction nucleases are enzymes that cut DNA at specific sites, usually a sequence of four to eight nucleotides. These different restriction nucleases protect the bacteria by degrading foreign DNA. The sequences identified by the restriction nucleases also occur in the genome of the bacteria itself, but the genome is protected from cutting by methylation. Foreign DNA is generally not methylated and therefore gets degraded by the nuclease (Alberts 2008).

Plasmid incompability

Incompability is the inability of two plasmids to stably coexist in the same cell. Incompability may be due to the sharing of one or more element of the replication or partitioning system, and loss of plasmids due to incompability is often a consequence of interference with the ability of the plasmid to correct random fluctuations in its copy number (Novick 1987). A number of incompatibility groups within plasmids have been recognized. Plasmids in one incompability group exclude each other from replicating in the same cell, but they can coexist with plasmids from other groups (Madigan, Martinko et al. 2006).

Materials and methods

E. coli strains

All clinical E. coli isolates were provided by professor Andrew Jenkins and Dr. Med. Nils Grude. The isolates were collected from samples sent to Unilabs Telelab, where they were identified as *E. coli* and resistant solely to mecillinam.

In total there were 11 isolates, all stored in a -70°C freezer.

Strains carrying plasmids pTB59 and pLP31 were provided by Piet de'Bohr.

Control strains JM109 provided by Promega (Madison, Wisconsin, USA) and 00157:97 provided by Karin Brekke Li, HiT.

Cultivation

All isolates were cultured at 37°C on LB-agar supplemented with antibiotics as appropriate. The concentrations were; ampicillin 100µg/ml, chloramphenicol 15µg/ml, mecillinam 80µg/ml (appendix 1). When liquid culture was needed the bacteria were cultivated in LB-broth at 37°C on a shaking platform (appendix 1). If not otherwise specified, the amount of liquid media was 50 ml supplemented with 50µl 20% glucose and 25µl mecillinam (80 mg/ml).

Growth curves

To create growth curves, 2 ml of overnight liquid culture was transferred into 50 ml of fresh liquid medium and incubated at 37°C on a shaking platform

To measure the bacterial growth "Spectronic 20" spectrophotometer (Bausch and Lomb, Antwerpen, Belgium) measuring optic density (OD) at 540 nm was used.

OD was measured every 20 minutes until the growth rate declined, approximately between 350 - 400 minutes.

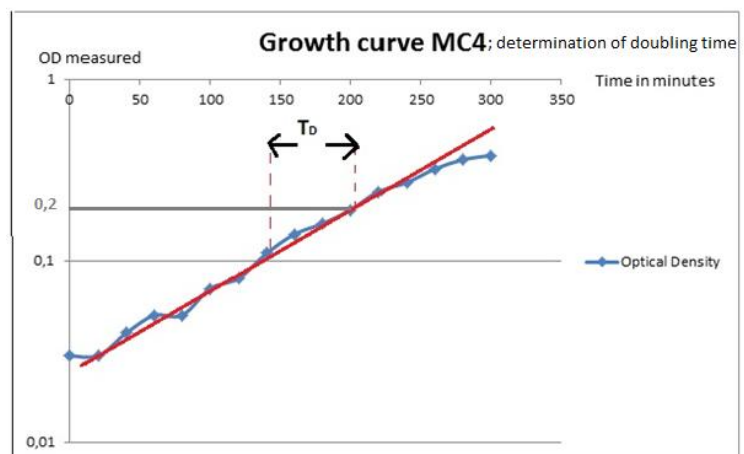Figure 11 shows how doubling time was calculated.

Figure 11: Shows how to calculate the doubling time. TD = 202 - 134 = 68 minutes.

Plasmids

Engineered pGEM®-3Z Vector (Promega, Madison, Wisconsin, USA) was used as a positive control in transfection experiments (appendix 2).

pTB59 and pLP31 plasmid DNA was prepared by the alkaline lysis method (Maniatis, Fritsch et al. 1982) (appendix 1). Maps of pTB59 carrying the *mrd* genes and pLP31 carrying the *mre* genes are shown in appendix 2 figures 36-37. No experiments with pLP31 were attempted.

In order to improve plasmid yield chloramphenicol enrichment was performed as described by Maniatis, Fritsch et. al, 1982.

Electroporation

For electroporation an electroporator 2510 (Eppendorf, Hamburg, Germany) was used.

1. 2 ml of overnight culture was added to 50 ml fresh liquid media, incubated at 37°C and harvested at mid-exponential growth phase

2. 1,5 ml aliquots of the culture were transferred to sterile Eppendorf tubes and harvested by centrifugation by centrifuge A14 (Jouan, st.Herblain, France) at maximum speed for 5 minutes

3. Media was poured off and the pellet resuspended in 100µl electroporation buffer

4. The mixture was centrifuged for another 5 minutes then resuspended in 100µl fresh electroporation buffer

5. 1 - 10µl of plasmid DNA was added

6. The mixture was transferred to an electroporation cuvette, taking care to avoid the formation of air bubbles

7. A 1500V electrical pulse was applied

8. The cuvette was removed from the electroporator, and 1,5 ml fresh medium was added before the liquid was transferred back to original tube.

9. The mixture was centrifuged at maximum speed for 1 minute and resuspended in 1 ml of fresh medium

10. Tubes were incubated at 37°C for one hour.

Steps 3 – 6 were performed on ice.

After electroporation, the bacteria were diluted 1:10 in 0,9% saline and spread on agar plates containing selective antibiotics.

The control, no plasmid added, was spread both on plates containing mecillinam to control for viability and on the plate selective for used plasmid (negative control).

Plates were incubated at 37°C, and examined after 24 - 48 hours.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to control the success of plasmid extraction and to verify plasmid size. For recipes of buffer and gel, see appendix 1. The agarose gel was run for approximately 24 hours at 15V. As size reference, λ-DNA ladder (Promega, Madison, Wisconsin, USA) both diluted 1:10 and undiluted were used. Plasmids were treated with restriction enzymes HindIII, EcoR1 (Promega, Madison, Wisconsin, USA) and BtgI (New England Biolabs, Ipswich, Massachusetts, USA) along with associated buffer. 1µl of enzyme plus 1µl of buffer was added to 5µl of plasmid preparation, total volume adjusted to 10 µl with distilled H_2O, and incubated for 30 minutes. Before the agarose gel was run, 2,5µl loading dye was applied to 5µl of each plasmid product and ladder. The substances were mixed using a pipette, and applied in to the different wells of the agarose gel.

After 24 hours the gel was stained with GelRed Acid Stain 10,000X in Water (Biotium, Hayward, California, USA) 100 µl/100 ml water for 30 minutes. The gel was then photographed using a GeneSnap gel documentation apparatus, and GeneSnap software (Syngene, Cambrigde, United Kingdom) was used to get a graphic processing of the results.

Resistance determination

Resistance determinations were performed using broth dilution, disc diffusion and well diffusion methods in order to investigate the effect of introducing the *mrd* genes of pTB59 on mecillinam resistance. IPTG was used as an inducer and glucose was used as a repressor.

All overnight culture were diluted to match a 0,5 McFarland turbidity standard (OD= 0,1) prior to further work.

Broth dilution test

Overnight cultures were diluted in 3 different media; LB, LB with glucose and LB with IPTG (For concentrations see appendix 1) and dispensed in the wells of a microtiter plate. A 4g/L solution of mecillinam was then serially diluted 1:2 through the bacterial suspension. The plates were incubated overnight on a shaking platform before results were read.

Disc diffusion and well diffusion tests

Resistance tests on agar plates were performed on dishes containing LB, LB + glucose and LB + IPTG (For concentrations see appendix 1). Both agar disk diffusion and agar well diffusion tests were performed by uniformly spreading culture on petri dishes (For the diffusion test bacterial suspension from colonies were used, while doing the well diffusion test overnight culture was used), then left to dry for about 10 minutes. Agar disk diffusion test were performed using Oxoid Mecillinam disks,

10µg. When the plates has dried two disks containing mecillinam were placed on the surface of the agar for the disk diffusion test. For the well diffusion test four wells were punched out of the agar using the broad end of a 1000 µl sterile pipette tip. These wells were then filled with either LB, LB containing respectively r 0.4 , 0.04 or 0.004 g/l mecillinam. Dishes were incubated at 37°C overnight before examined.

Recombination test

In order to see whether normal colony morphology was restored by recombination between pTB59 and the chromosome of MC3, recombination test were performed. The test was performed on MC3, MC3::pTB59 1 and MC3::pTB59 4. MC isolates from freeze culture were spread on selective media and incubated over night before 5 separate colonies from each isolate were spread on non-selective plates (LB + glucose, for concentrations see appendix 1) and incubated at 37°C overnight before plates were examined for presence of normal sized colonies.

Microscopy

To get a visual of the bacteria, strains were viewed under a CX22LED microscope (Olympus, Hamburg, Germany) at 100x magnification using a coverslip and oil immersion. Unstained wet preparation from overnight broth cultures was used.
Pro Microscan 5898 (Oplenic Optromics Hangzhou, China) USB camera along with ScopePhoto (ScopeTek, Hangzhou, China) software was used to capture pictures of the bacteria.

Results

Colony morphology

 MC-isolates grown on agar plates cultivated for 24 hours showed growth of small or very small colonies, 3 of the 11 colonies appeared mucoid (MC1, MC2 and MC7).
All isolates were slow growing, as seen in figures 13 - 23.

Cell morphology

Figure 12 clearly shows the abnormal cell shape of MC3; the cells appear round instead of the normal rod shape of *E. coli*.
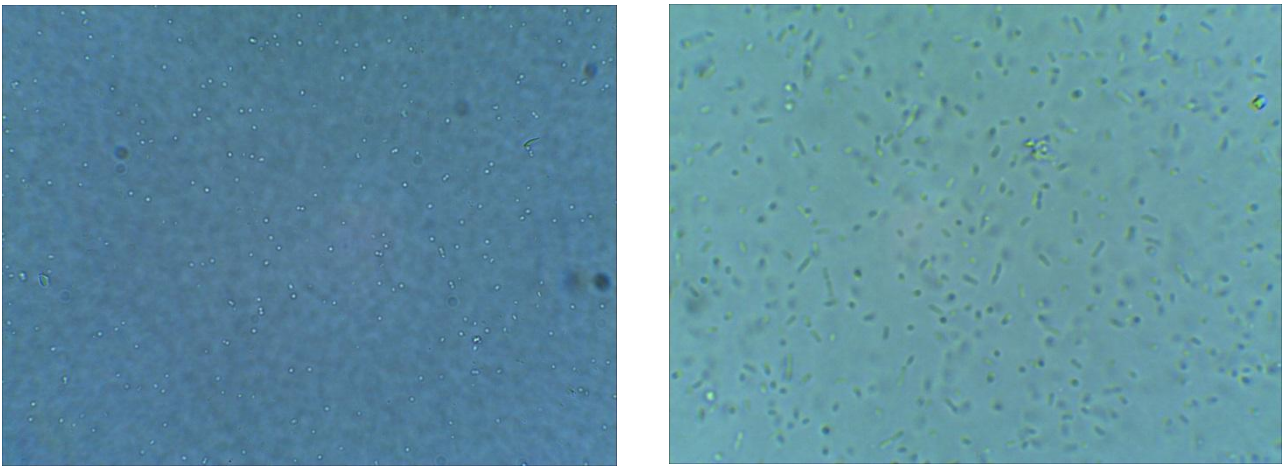

Figure 12: Micoroscopic photograph of MC3 to the left, clearly showing a round cell shape. Normal rod shaped *E. coli* strain 00157:97 to the right.

Doubling time

Doubling time was estimated from growth curves (see figures 13- 23).Times for the isolates were 54 - 94 minutes, with an average of 80 minutes. This is shown in table 1.

Table 1 : Shows the doubling time of all MC isolates.

Culture	Time period used	T_D
MC1	220 - 140	80
MC2	194 - 106	88
MC3	243 - 160	83
MC4	202 - 134	68
MC5	152 - 72	80
MC7	172 - 88	84
MC9	240 - 150	90
MC10	234 - 140	94
MC11	175 – 100	75
MC12	104 - 50	54
MC13	120 – 33	87

Growth curves

Growth curves for the MC-isolates shows all strains to be slow growing compared to normal *E. coli*. The curves also shows us mid-exponential growth phase, which is essential in association to the harvesting of cells for electroporation (figures 13 - 23). Some of the growth curves , like MC1 (fig 13) and MC7 (fig 18),show quite a compex shape compared to classical growth curves, and this might make the estimation of the doubling time somewhat uncertain.
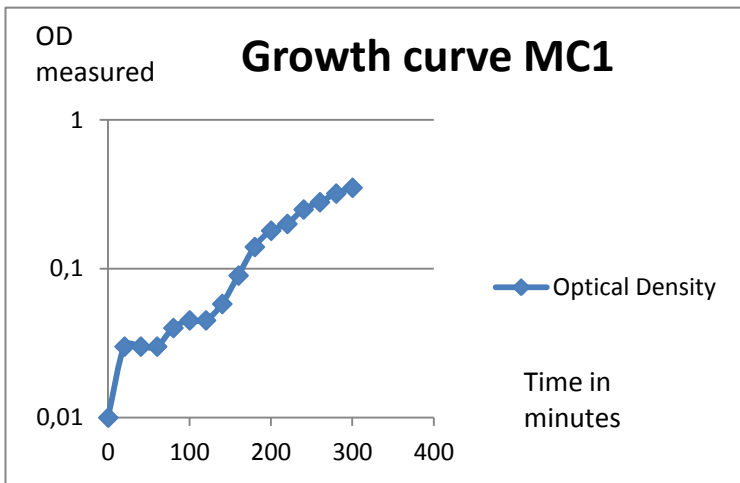

Figure 13: growth curve for MC1
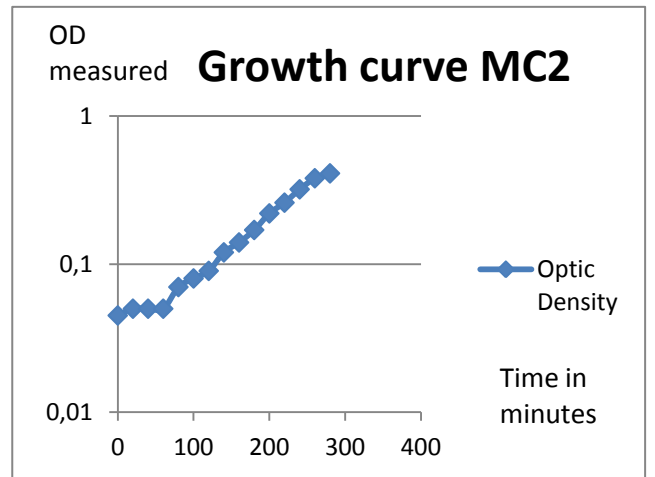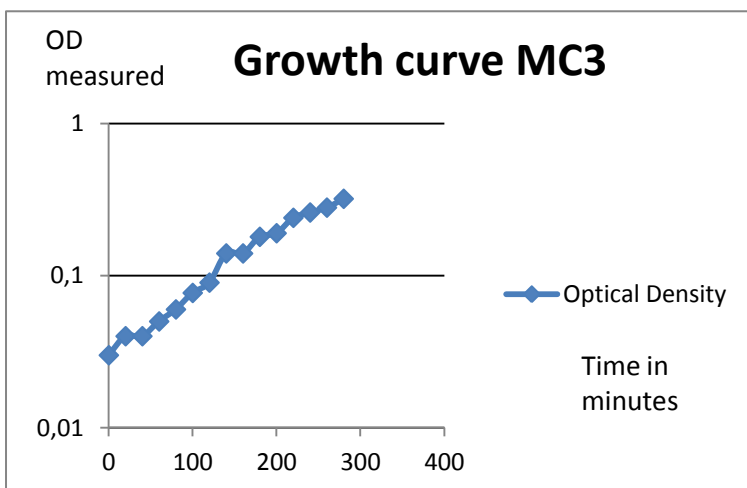

Figure 14: Growth curve for MC2
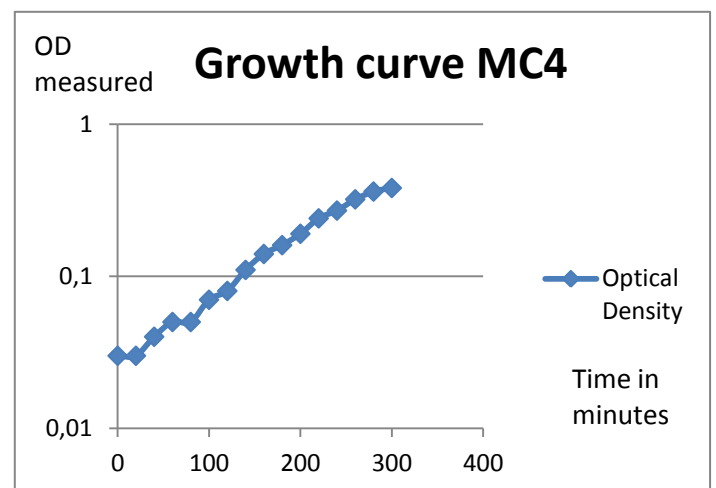

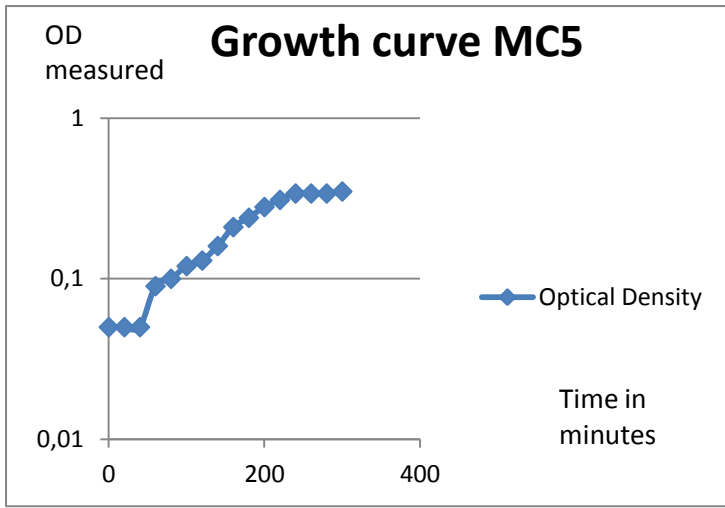Figure 15: Growth curve MC3

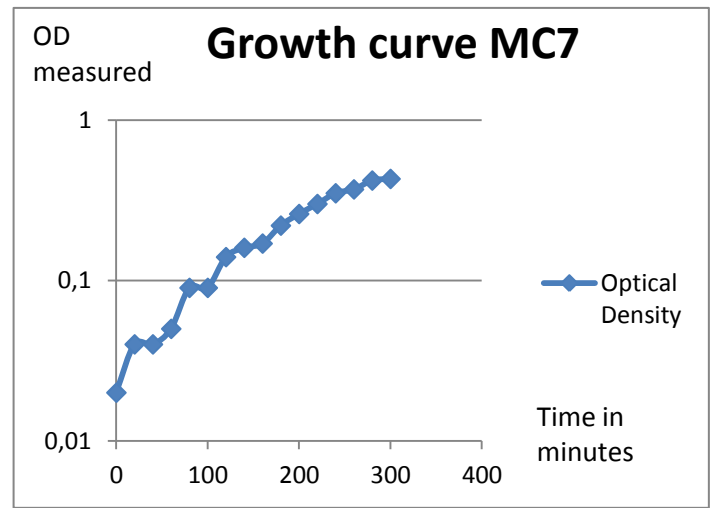Figure 16: Growth curve MC4

Figure 17: Growth curve MC5
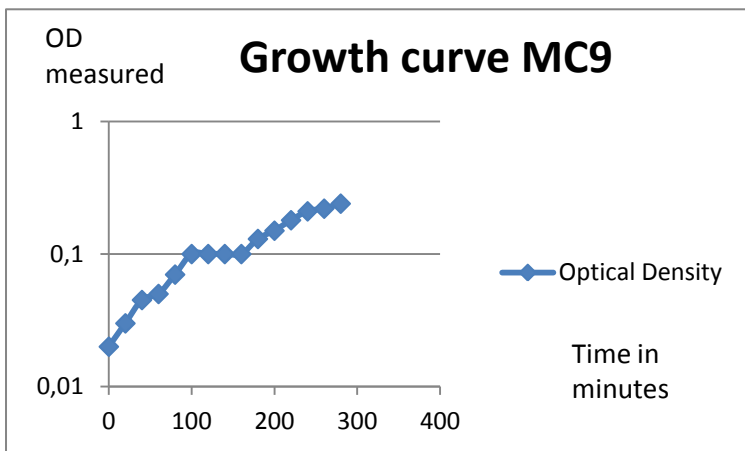

Figure 18: Growth curve MC7
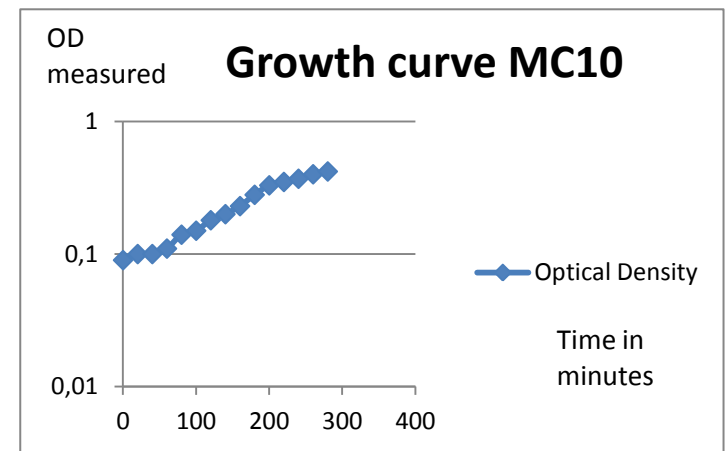

Figure 19: Growth curve MC9
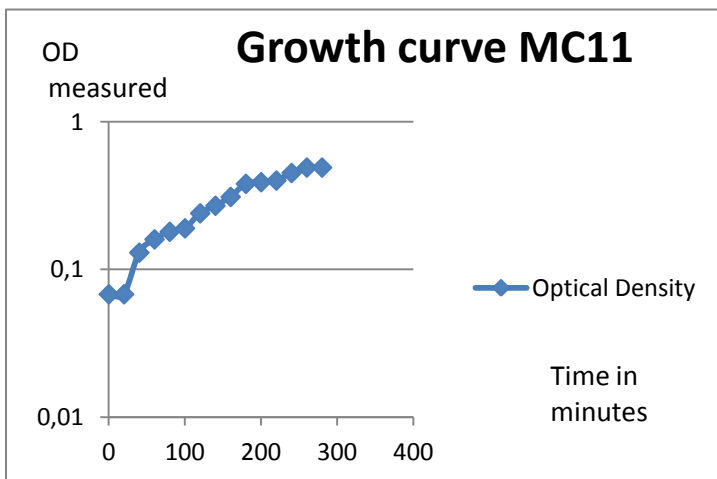

Figure 20: Growth curve MC10
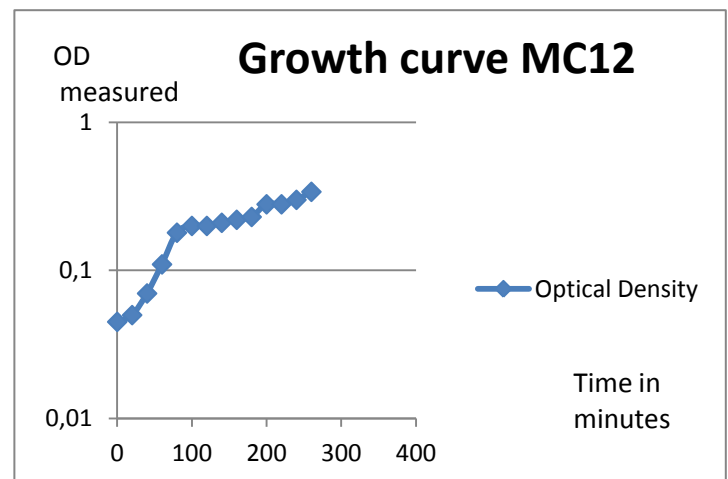

Figure 21: Growth curve MC11
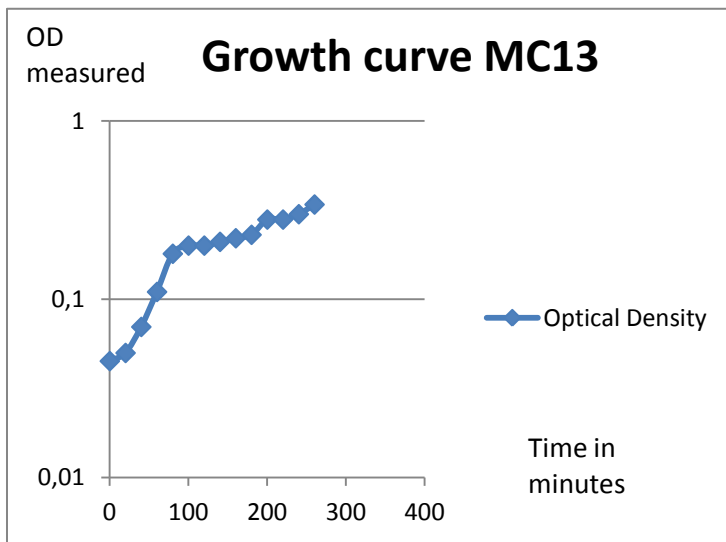

Figure 22: Growth curve MC12

Figure 23: Growth curve MC13

Electroporation and agarose gel electrophoresis

Electroporation trials were run approximately 70 times in total and performed on all 11 isolates, pTB59 and pGEM®-3Z plasmid preparations were used.

Sucessfull electroporation of MC3

Only one electroporation trial was successful: MC3 + pTB59. Four colonies grew on selective ampicillin plates. Samples from these colonies were spread on new ampicillin plates. Only two of these colonies could be successfully cultivated on ampicillin plates. These are called MC3::pTB59-1 and MC3::pTB59-4 (Fig. 24). Samples from each colony were cultured overnight before plasmid DNA was isolated and treated with HindIII, EcoR1 and BtgI then run through agarose gel electrophoresis to establish that the bacteria actually were pTB59 transformants (Fig. 25).
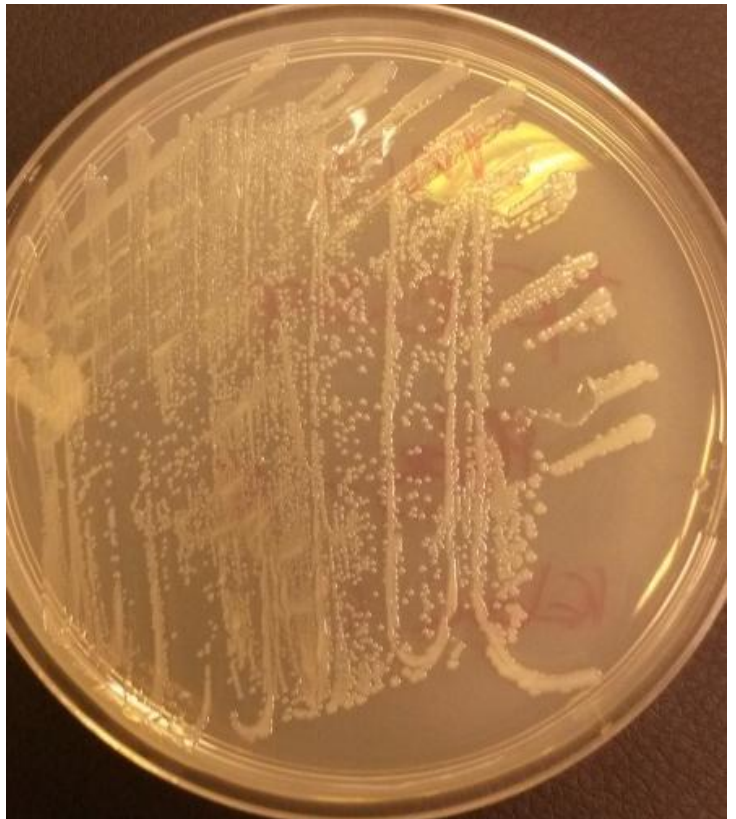

Figure 24: MC3::pTB59 1 transformant

The agarose gel electrophoresis showed that MC3 had incorporated the pTB59 plasmid (Fig. 25 and table 2).

Well	DNA	Amount	Restriction Enzyme	Amount
1	λ ladder	5 µl		
2	λ ladder 1:10	5 µl		
3	MC3::pTb59 1	5 µl		
4	MC3::pTB59 1	5 µl	HindIII	1µl
5	MC3::pTB59 1	5 µl	EcoR1	1µl
6	MC3::pTB59 1	5 µl	HindIII+EcoR1	1µl
7	MC3::pTB59 1	5 µl	BTG1	1µl
8	pTB59 4	5 µl		
9	MC3::pTB59 4	5 µl	HindIII	1µl
10	MC3::pTB59 4	5 µl	EcoR1	1µl
11	MC3::pTB59 4	5 µl	HindIII+EcoR1	1µl
12	MC::3pTB59 1	5 µl	BTG1	1µl
13	Original pTB59 1	5 µl		
14	Original pTB59 1	5 µl	EcoR1	1µl
15	Original pTB59 1	5 µl	HindIII+EcoR1	1µl
16	Original pTB59 2	5 µl	EcoR1	1µl
17	Original pTB59 2	5 µl	HindIII+EcoR1	1µl

Figure 25: Agarosegel containing pTB59 from the MC::pTB59 transformants. For contents see table 2.

Investigation of MC3 transformants

To establish if the MC3::transformants possess different properties than the MC isolates, growth curves for both transformants were made. Growth curves performed on MC3::pTB59 1 and MC3::pTB59 4 showed a more rapid growth for the transformants than for the original MC3 isolate, this is shown in figures 26 - 28 , where the transformants are compared to the original MC3 isolate. Calculated doubling time for the transformants can be seen in table 2.

Table 3: Shows the doubling time for all MC isolates including MC3::pTB59 transformants.

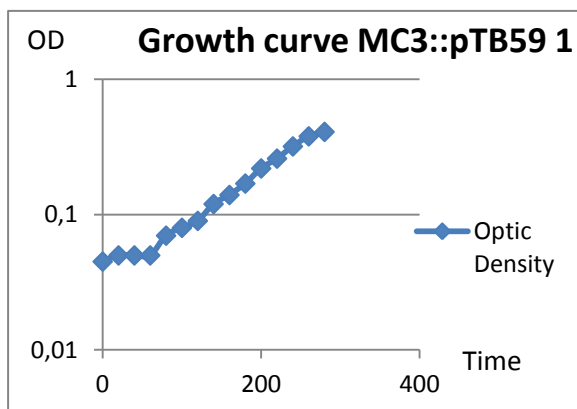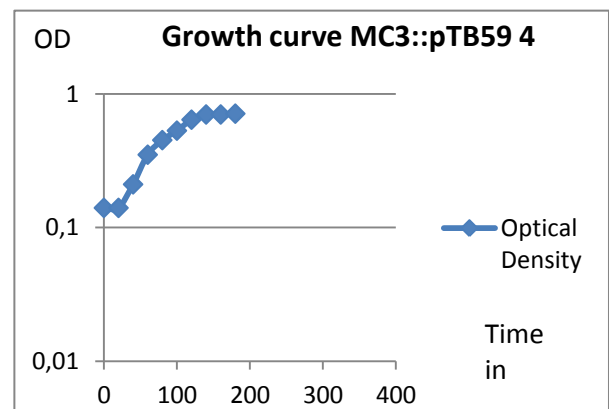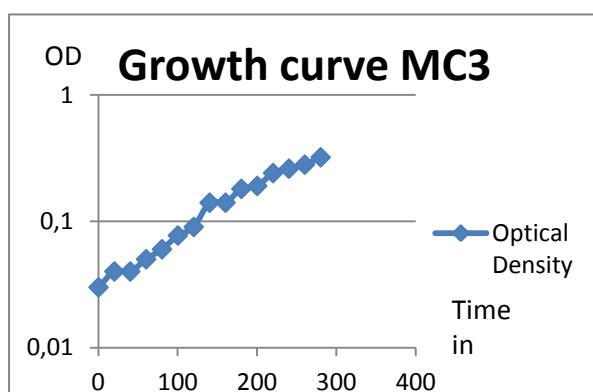| Culture	Time period used	T_D
MC1	220 - 140	80
MC2	194 - 106	88
MC3	243 - 160	83
MC4	202 - 134	68
MC5	152 - 72	80
MC7	172 - 88	84
MC9	240 - 150	90
MC10	234 - 140	94
MC11	175 – 100	75
MC12	104 - 50	54
MC13	120 – 33	87
MC3::pTB59 1	78 - 25	55
MC3::pTB59 4	105 - 55	50

Figure 26: Growth curve MC3::pTB59 1

Figure 27: Growth curve MC3::pTB59 4

Figur 28: Growth curve for MC3

33

A microscopic visual of the transformants were also performed. Control strain *E. coli* 00157:97 shows normal rod shape (Fig. 30) while MC3, MC3::pTB59 1 and MC3::pTB59 4 shows abnormal rounded morphology (Fig. 29). The use of the inducer IPTG did not cause any changes in the morphology (Fig. 31).

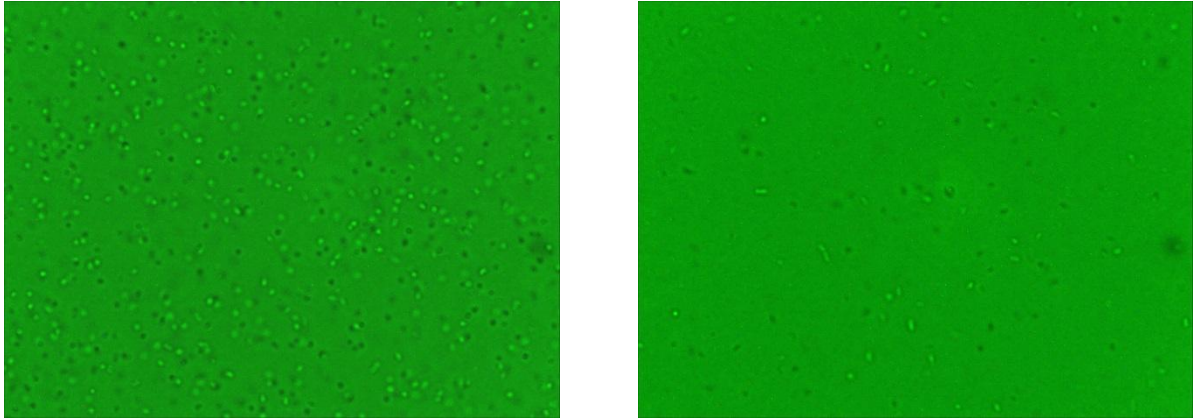**Figure 29: MC3::pTB59 1 to the left and MC3::pTB59 4 to the right cultivated in ampicillin + glucose (appendix 1). Both showing rounded morphology.**

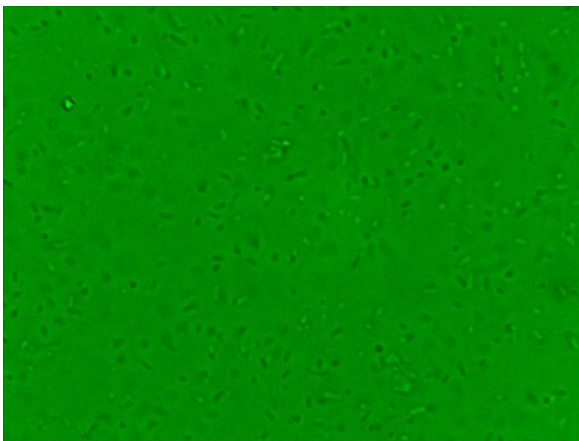

Figure 30: *E. coli* 00157:97 with normal rod shaped morphology.
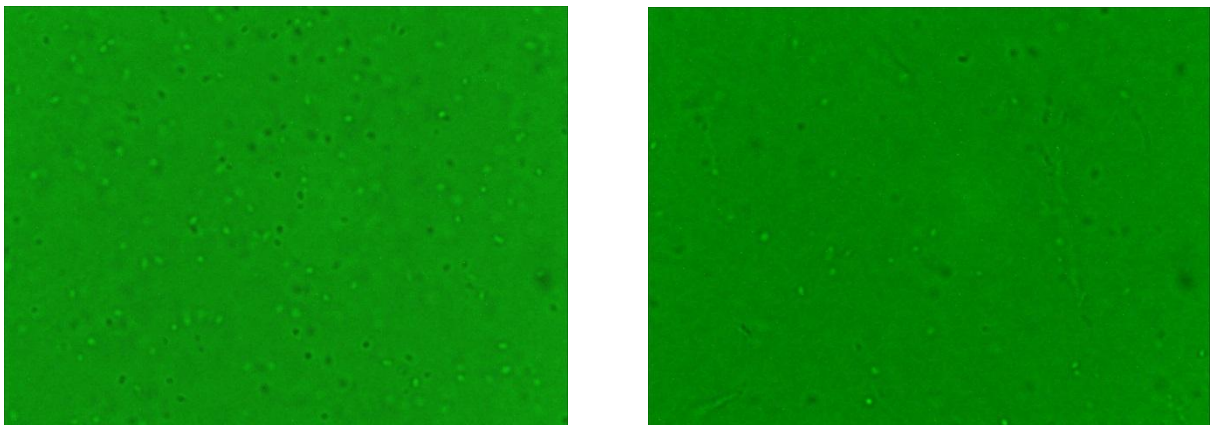
Figure 31: MC3::pTB59 1 to the left and MC3::pTB59 4 to the right cultivated with IPTG and ampicillin (appendix 1), still showing abnormal rounded morphology.

MIC - Minimal inhibitory concentration tests

Broth dilution test

The results of a typical broth dillution test is shown in figure 32.

On all plates only MC3 showed sensibility, with a mecillinam concentration of 2g/l (Fig. 32).

Microtiter plates were performed on MC3, MC3::pTB59 1 and MC3::pTB59 4 with regards to mecillinam, mecillinam + glucose and mecillinam + IPTG (appendix 1).

Figure 32: Rows A-C: MC3; rows D-H: MC3::pTB59. Inhibition as shown by absence of turbidity is seen in wells A1, B1 and C1.

Disc diffusion and well diffusion tests

Neither the agar disc diffusion test nor the agar well diffusion test showed any mecillinam sensibility for either MC3 or the MC3 transformants (Fig. 33). IPTG and glucose has no effect as inducer or repressor.
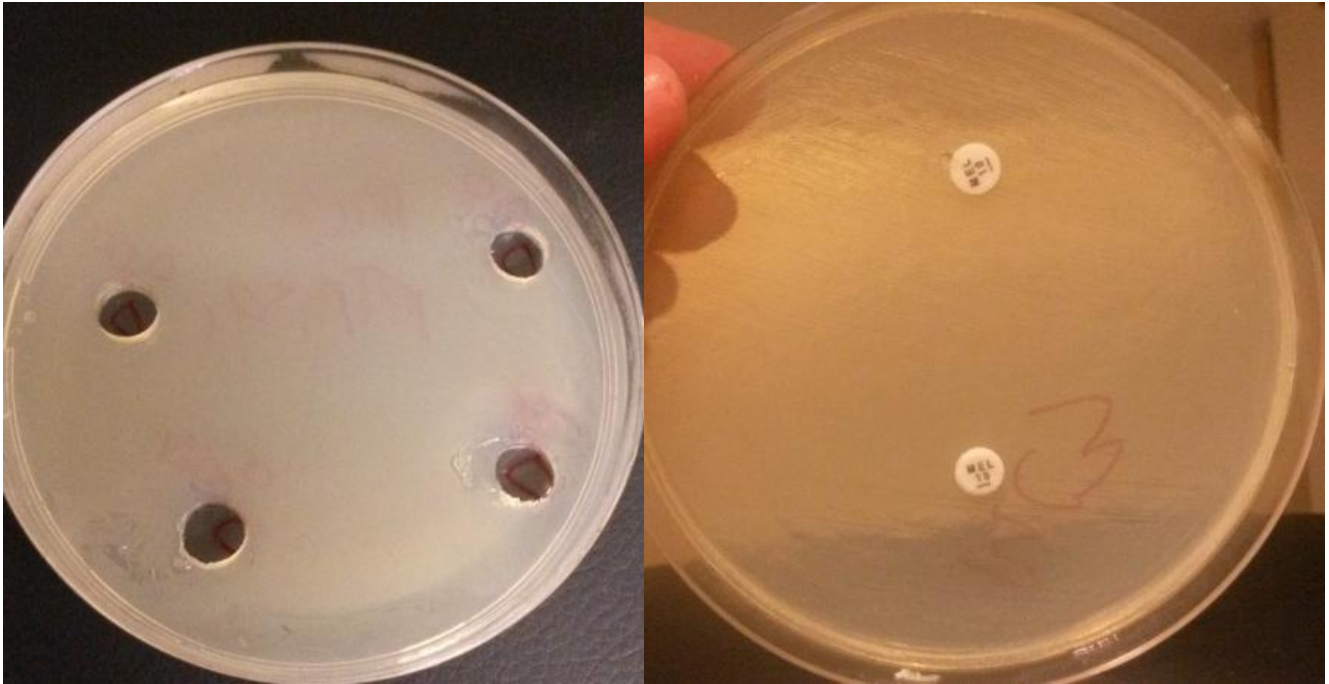
Figure 33: Agar disc diffusion test and agar well diffusion test showed no sensitivity.

Recombination test

The recombination test showed negative results. All populations on all 15 plates seems homogenous, so eventual recombination has not lead to any changes in phenotype.

The colonies on selective media appear smaller than the ones on non-selective media (Fig. 34).
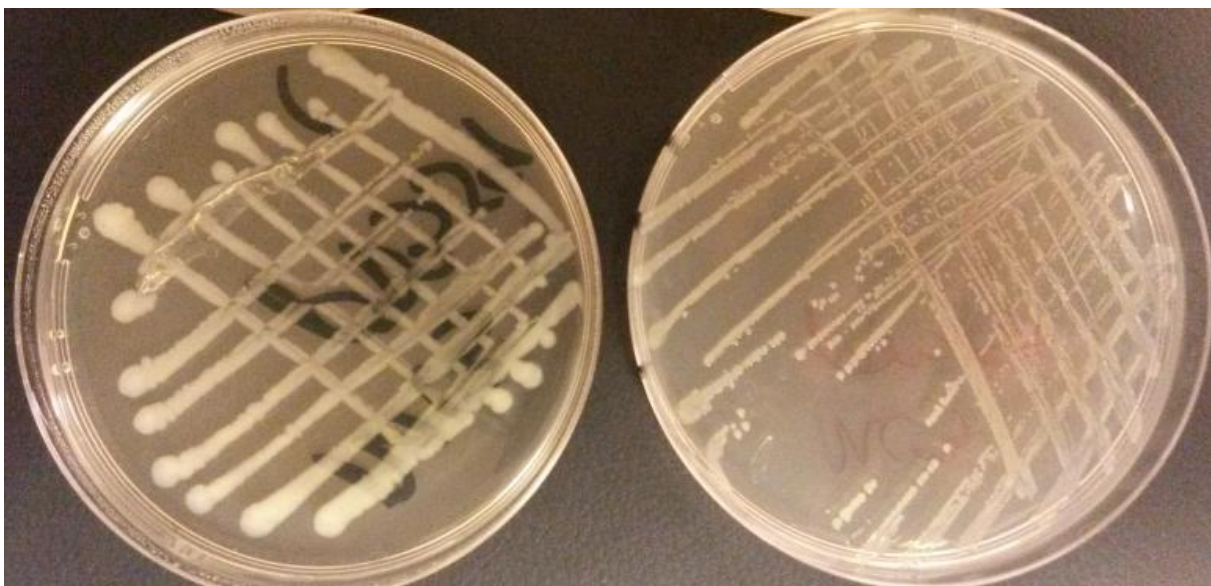

Figure 34: The recombination test showed homogenous growth.

Discussion

In this study I have investigated the properties of eleven mecillinam resistant clinical isolates of *Escherichia coli* whose properties suggest that they may be mutants at the *mrd* or *mre* loci. The isolates show abnormal cell morphology and small and/or abnormal colony size.

On order to test whether the isolates were *mrd/mre* mutants, I intended to introduce plasmids containing normal *mre* and *mrd* loci in order to see wether they would reverse these changes. Unfortunately due to technical difficulties and lack of time it was not possible to conduct any experiments with the plasmid carrying the *mre* gene.

Growth rates

Regarding to generation time, my results show a significant slower growth for the MC isolates grown in the laboratory compared to normal *E. coli* strains. Generation time for the MC isolates is calculated to be 54 - 94 minutes; normal *E. coli* grown in laboratories usually have a doubling time of 15 - 20 minutes (Todar 2004). Only MC12 shows a growth rate approaching normal, with a doubling time of 54 minutes. This indicates that my clinical isolates are slow growing, and not as healthy as normal *E. coli* strains. This would also explain the small size of the colonies, although MC12, which has the fastest growth rate, produced tiny colonies.

Investigation of MC3 and MC3 transformants

Successful electroporation of the plasmid pTB59 carrying the *mrd* genes was achieved only with MC3 and the discussion below refers to this strain alone.

The two MC transformants appear to show a more rapid growth, with a doubling time of 55 (MC3::pTB59 1) and 50 (MC3::pTB59 4) minutes . The original doubling time for the MC3 isolate was 83 minutes.

However, the MC3::pTB59 transformants showed a uniformly coccoid morphology indistinguishable from MC3 and their MIC for mecillinam was even higher than that of MC3.

Results of MIC measurements and cell morphology indicate that pTB59 does not complement the mutation in MC3, therefore MC3 is not a *mrd* mutant. Inducing *mrd* expression with IPTG or suppressing it with glucose had no effect on the phenotype.

If MC3 was an *mrd* mutant I would expect morphology approaching a normal rod shape, mecillinam sensitivity and larger colony sizes in addition to higher growth rates. The rapid growth of the transformants might be explained with the use of a different shaking platform, providing a higher oxygen transfer rate which in turn increases cell growth rate.

If MC3 is an *mrd* mutant, pTB59 should be able to correct the chromosomal mutation by recombination, resulting in a normal phenotype in some colonies even without induction with IPTG and the lack of glucose. The recombination test showed all populations on all 15 plates homogenous, so eventual recombination did not lead to any changes in phenotype. The colonies on selective media seem smaller than the ones on non-selective media, but this is probably caused by stress in the populations when antibiotics are present.

All strains tested for resistance (MC3, MC3::pTB59 1 and MC3::pTB59 4) shows a high tolerance for mecillinam, also the MC3 transformants. Both agar well diffusion and agar disk diffusion test showed no sensitivity towards mecillinam, regardless of which media was used; induction of *mrd* with IPTG did not make any visible reduction in mecillinam resistance. The MC3 transformants grew even with as high mecillinam concentration as 4g/L, the MC3 isolate at 2g/L. Previous lab tests found MIC for MC3 = 128/256 g/L (Andrew Jenkins 2014, pers. comm.). The difference in resistance might be due to use of LB rather than Mueller-Hinton media. It is also possible that the increased resistance is caused by the activity of the β-lactamase gene of pTB59. A Clover-leaf test could be run to investigate this further (Orstavik and Odegaard 1971).

The pTB59 plasmid encodes for the *mrd* region and since *mrd* does not complement MC3, MC3 is not an *mrd* mutant. MC3 could be an *mre* mutant, probably in PBP2, which could be investigated by introducing pLP31. Another possibility is that MC3 is neither an *mrd* or *mre* mutant, and the study hypothesis is incorrect.

With exception of MC3 none of the MC isolates could be successfully electroporated. This is discussed below.

In all cases the bacteria grew normally on mecillinam plates after electroporation, therefore surfactant detergent residues, factors that affect the conductivity that again affect f. ex the buffer-solutions used, too poor routines, use of too old buffer-solutions etc. are not likely explanations, as these are likely to impact viability. Electroporation conditions were established with strain JM109 and it is not necessarily the case that they are optimal for the MC strains. It may therefore be necessary to optimize electroporation conditions for each strain.

Did the abnormal cell shape inhibit electroporability?

I have not been able to find literature describing electroporation of morphologically abnormal *E. coli* strains, neither electroporation were the pTB59 plasmid has been used, it is therefore difficult to draw any conclusions on this possibility.

Do the MC strains have mechanisms that inhibit establishment of the introduced plasmid? If the MC isolates already contain plasmids, that are incompatible with the pTB59 plasmid they may prevent its establishment. Two plasmids that cannot stably coexist in the same cell are said to be incompatible.

The MC isolates might also contain restriction nucleases that cleave the pTB59 plasmid and degrade it.

It is possible that the plasmid concentration was too low to give detectable transformation. In total I ran 12 repeated electrophoresis experiments (data not shown), because there were problems getting enough plasmid yield to read any results of the gel, and this implies a low concentration of plasmids in solution.

The possibility that the plasmids are actually successfully electroporated but their ampicillin resistance genes are not expressed does not seem very likely, as the genes we try to introduce are genes originated from *E. coli* itself.

Alternative approaches

Despite of the lack of success introducing plasmids to the MC isolates, considering the operating hypothesis I still would expect to find mutations in the *mre* and/or *mrd* regions. If this occurs as predicted, it is necessary to identify the type of mutation.

If the operating hypothesis was to be verified, I would expect to find either a frameshift, nonsense or a deletion mutation in the clinical MC isolates – mutations that have serious consequences for the gene expression. This could be investigated by DNA sequencing.

The *mre* region is located at 71 min and the *mrd* region at 14 min on the *E. coli* chromosomal map (Wachi, Doi et al. 1987). By identifying and sequencing the *mre* and *mrd* regions of the bacteria, these regions can be compared to the same regions in normal *E. coli* strains, and this way mutation can be identified.

Hopefully indentification of the predicted mutations would lead to an understanding of the observed resistance towards mecillinam in the clinical MC isolates.

Also introduction of F-primes via conjugation or λ-transducing phages by transduction could be a possible approach to introduce desired genes into the MC strains to investigate if the strains lack properly functional genes within the suspected *mre* or *mrd* regions.

References

Alberts, B. (2008). Molecular biology of the cell. New York, Garland Science.

Bendezu, F. O. and P. A. de Boer (2008). "Conditional lethality, division defects, membrane involution, and endocytosis in mre and mrd shape mutants of Escherichia coli." J Bacteriol **190**(5): 1792-1811.

Cabeen, M. T. and C. Jacobs-Wagner (2005). "Bacterial cell shape." Nat Rev Microbiol **3**(8): 601-610.

Clarke, P. D., A. M. Geddes, D. McGhie and J. C. Wall (1976). "Mecillinam: a new antibiotic for enteric fever." Br Med J **2**(6026): 14-15.

Ghuysen, J. M. and R. Hackenbeck (1994). Bacterial cell wall. Amsterdam ; New York, Elsevier.

Grude, N. (2006). Urinary tract infections with emphasize on bacterial etiology, sensitivity, virunelce and clonal distribution. 1 PhD, University of Tromsø.

Leo Pharma, AS (2008). Selexid Leo - i dagens behandling av urinveisinfeksjoner (UVI). LEO Pharma, AS. Norway**:** 10.

Low, K. B. (1972). "Escherichia coli K-12 F-prime factors, old and new." Bacteriol Rev **36**(4): 587-607.

Madigan, M. T., J. M. Martinko and T. D. Brock (2006). Brock biology of microorganisms. Upper Saddle River, NJ, Pearson Prentice Hall.

Maniatis, T., E. F. Fritsch and J. Sambrook (1982). Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory.

Miklavcic, D., L. M. Mir and P. Thomas Vernier (2010). "Electroporation-based technologies and treatments." J Membr Biol **236**(1): 1-2.

Mims, C. A. (2004). Medical microbiology. Edinburgh ; New York, Mosby.

Neu, H. C. (1983). "Penicillin-binding proteins and role of amdinocillin in causing bacterial cell death." Am J Med **75**(2A): 9-20.

Neu, H. C. (1985). "Amdinocillin: a novel penicillin. Antibacterial activity, pharmacology and clinical use." Pharmacotherapy **5**(1): 1-10.

Novick, R. P. (1987). "Plasmid incompatibility." Microbiol Rev **51**(4): 381-395.

Orstavik, I. and K. Odegaard (1971). "A simple test for penicillinase production in Staphylococcus aureus." Acta Pathol Microbiol Scand B Microbiol Immunol **79**(6): 855-856.

Pierce, B. A. (2002). Genetics : a conceptual approach.

Schleif, R. (2000). "Regulation of the L-arabinose operon of Escherichia coli." Trends Genet **16**(12): 559-565.

Seltmann, G. and O. Holst (2001). The bacterial cell wall. Berlin ; New York, Springer.

Tortora, G. J., B. R. Funke and C. L. Case (2007). Microbiology : an introduction. San Francisco, Pearson Benjamin Cummings.

Tybring, L. and N. H. Melchior (1975). "Mecillinam (FL 1060), a 6beta-amidinopenicillanic acid derivative: bactericidal action and synergy in vitro." Antimicrob Agents Chemother **8**(3): 271-276.

Wachi, M., M. Doi, S. Tamaki, W. Park, S. Nakajima-Iijima and M. Matsuhashi (1987). "Mutant isolation and molecular cloning of mre genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in Escherichia coli." J Bacteriol **169**(11): 4935-4940.

Weaver, J. C. and Y. A. Chizmadzhev (1996). "Theory of electroporation: A review." Bioelectrochemistry and Bioenergetics **41**(2): 135-160.

Appendix 1

Liquid media

Luria-Bertani medium

Per. Liter:

950 ml deionized H20 add:

Bacto –tryptone 10 g

Bacto yeast extract 5 g

NaCl 10g

pH adjusted to 7.0 using 5M NaOH.

Freeze media

Add 20% glycerol to liquid media

Agar

Add 15g/l bacto agar to liquid media before autoclaving.

All media were autoclaved at 121°C for 20 minutes.

Selective media

For petri dishes add one of the following after autclaving:

Mecillinam: 0,25 ml/250 ml agar

Chloramphenicol: 0,5 ml/500 ml agar

Ampicillin: 0,5 ml/ 500 ml agar

Concentration of antibiotics:

Ampicillin 100µl/ml

Chloramphenicol 15µg/ml

Mecillinam 80µg/ml

Glucose 0,5% solution

IPTG concentration of 50 µM

Plasmid solutions – lysozyme buffers

→ Solution I

50mM glucose

25mM TrisHCl pH 8,0

10mM EDTA pH 8,0

adjust pH to 8,0 using HCl

→Solution II

10 ml 0,2M NaOH

1% SDS

→Solution III

60 ml 5M potassiumacetat (0,5 g /100 ml vann)

11,5 ml glacial acetic acid

28,5 ml H_2O

Electroporationbuffer – EPB

17,1 g sucrose

2 ml $MgCl_2$ 25mM (0,5g/100ml)

7 ml Potassium Phosphate (KP)-buffer 100mM pH 7,4

→H_2O opp til 100 ml

TAE-buffer

TAE-buffer 50 x concentrate, 20 ml to 1 l of water

Agarosegel

100 ml TAE-buffer

1 g agarose

= 1% agarose

Autoclaved for 20 minutes at 121°C, then cooled to about 50°C, then molded in to shape.
Wait about 30 minutes for the gel to solidify.

pH

To adjust the pH of the different solutions MeterLab PHM210 standard pH meter (Radiometer
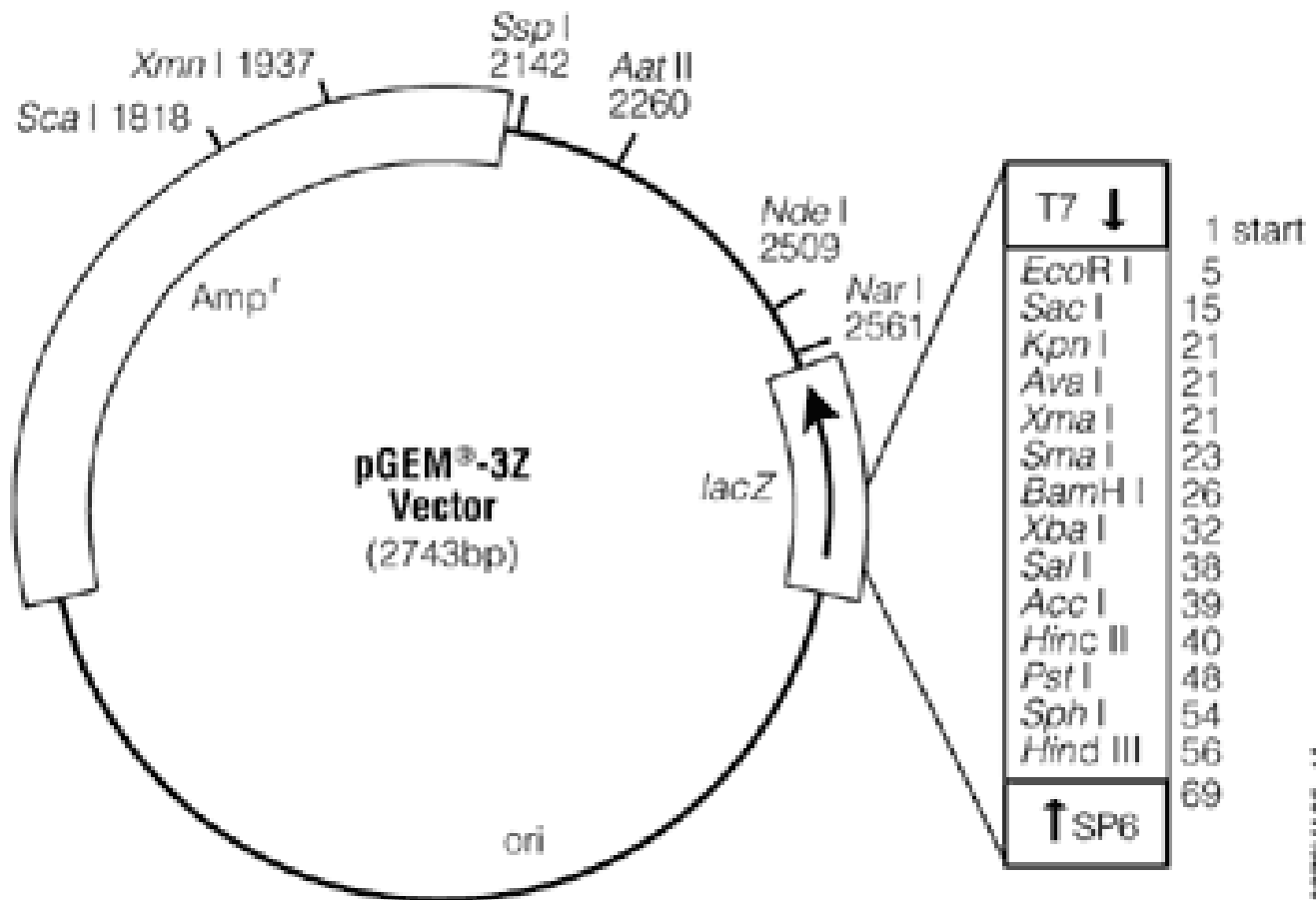analytical SAS, Villeurbanne Cedex, France) were used.

Appendix 2

Figure 35: pGEM®-3Z Vector (Promega, Madison, Wisconsin, USA)

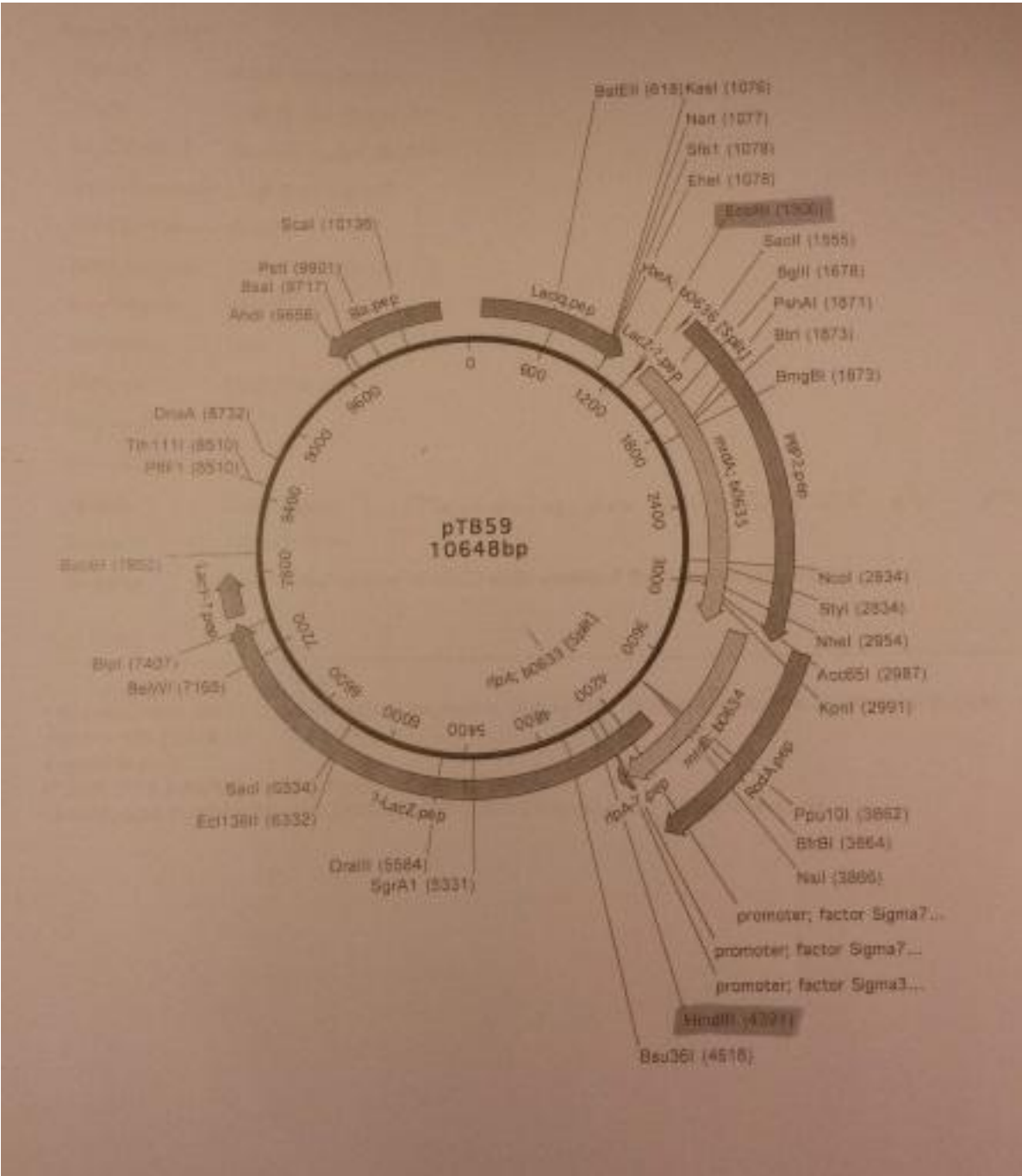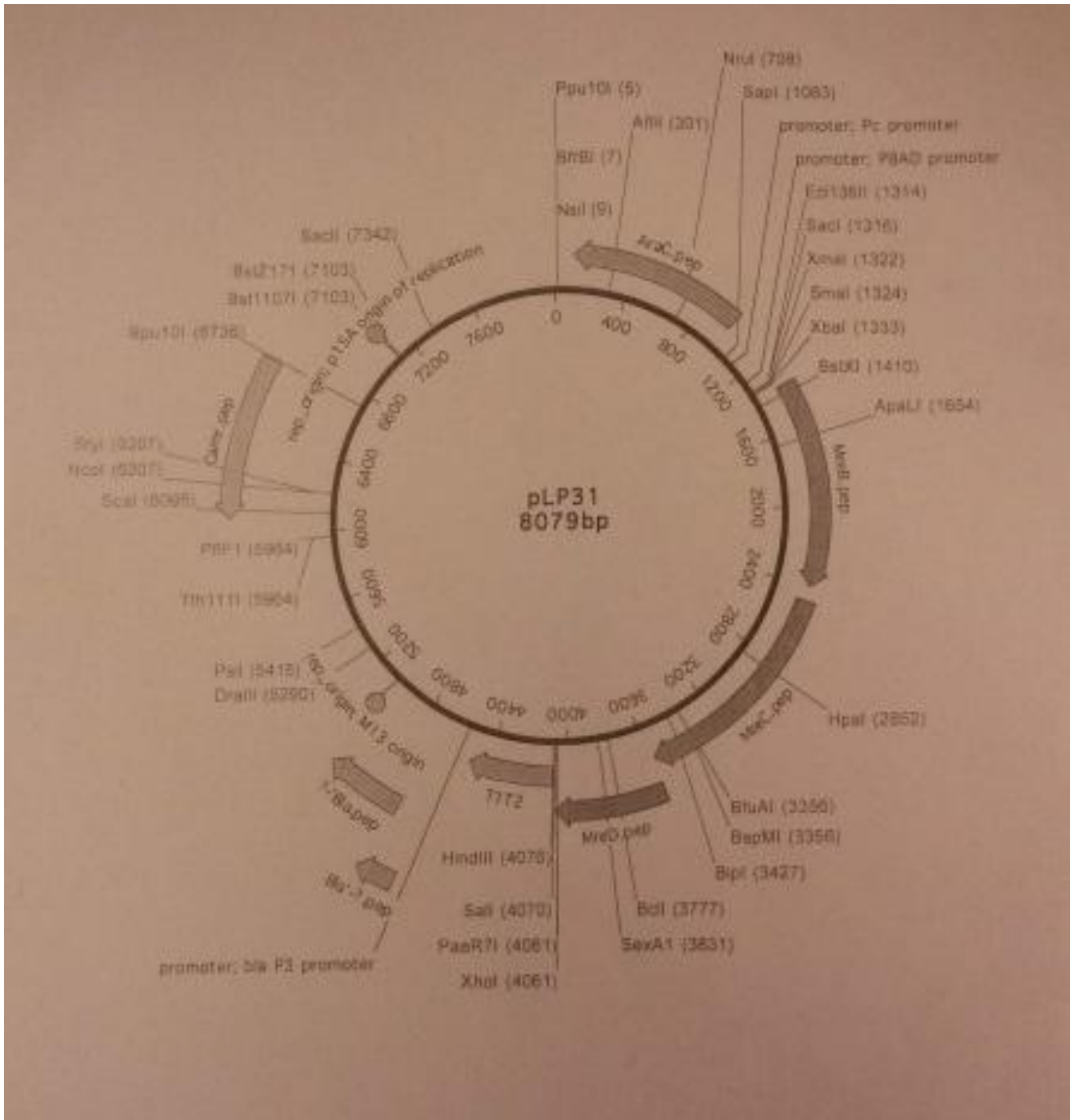http://no.promega.com/~/media/images/resources/figures/0200-0299/0278vaw4.gif?la=en

Figure 36: genetic map pTB59

Figur 37: genetic map pLP31