The Role of Clay Minerals in the Transformation of E. coli

Ekaterini Porttellou
CUNY City College

2012

Recommended Citation
http://academicworks.cuny.edu/cc_etds_theses/150

How does access to this work benefit you? Let us know!
Follow this and additional works at: http://academicworks.cuny.edu/cc_etds_theses
Part of the Biology Commons

This Thesis is brought to you for free and open access by the City College of New York at CUNY Academic Works. It has been accepted for inclusion in Master's Theses by an authorized administrator of CUNY Academic Works. For more information, please contact AcademicWorks@cuny.edu.
The Role of Clay Minerals in the Transformation of *E. coli*

submitted by Ekaterini Portellou in partial fulfillment of the Master of Arts degree

Faculty Advisor: Professor Karin A. Block
Abstract: The bacteria *Escherichia coli* and plasmid DNA PUC18 were suspended with the clay mineral montmorillonite to explore their interaction and the role of the clay to act as a reservoir for DNA. PUC18 plasmid isolation was confirmed by the restriction enzyme PVUII. The hypothesis of whether DNA bound to montmorillonite has the ability to transform competent cells and therefore transfer genetic material was tested. The activity of clay-bound DNA in the presence of the restriction enzyme EcoRI was also investigated to determine if degradation would occur as it happens naturally in free DNA or if it would be protected in some extent due to the presence of clay. In addition, homoionic (Mg-substituted) clay was suspended and incubated with DNA to determine if the adsorption would also occur in the interlayer as well as at the edges of the clay as expected by using X-ray diffraction. Experiments revealed that increasing the mass of clay while keeping the amount of DNA constant and vice versa resulted in successful transformation of competent cells. However, whether inhibition occurred could not be conclusively determined. Furthermore, the adhesion and binding mechanisms still need to be explored to conclude that montmorillonite acts as a reservoir or delivery vehicle for DNA. DNA bound to montmorillonite was protected from EcoRI restriction endonuclease suggesting that DNA, once adsorbed to montmorillonite, is resistant to degradation caused by the action of the restriction enzyme and allows it to transform. X-ray diffraction data indicated no significant change in the basal spacing of the clay with bound DNA suggesting that the adhesion of DNA molecules did not take place in the interlayer.
Acknowledgement

I would like to acknowledge and extend my heartfelt gratitude to the following persons who have made the completion of this thesis possible: my mentor, Dr. Karin Block, for her vital encouragement, support and advice during this stressful period, Dr. Paul Gottlieb, for his assistance and understanding. I would also like to especially thank Alexandra Alimova for all her help and taking time from her busy schedule to guide me through all the experiments. Hui Wei for his selflessness and help and the faculty and staff of the EAS Department of Science of the City College of New York. Most especially, I must thank my father and mother, whose sacrifice and commitment to my education made all of this possible. To my family and friends, too numerous to name: Thank you.
Index

Abstract........................................................................................................ii
Acknowledgment......................................................................................iii
Index...........................................................................................................iv-v

1. Introduction..........................................................................................1-2

2. Background.........................................................................................3-15

3. Materials and Methods
   3.1 Bacteria Plasmids and Restriction Enzymes....................................16-17
   3.2 Preparation of Competent E. coli Cells...........................................17
   3.3 Preparation of Clay.........................................................................18-19
   3.4 Purification of Plasmid PUC18.........................................................19
   3.5 Preparation of Agarose Mini Gel....................................................19
   3.6 Plasmid Cut with Restriction Enzyme PVUII.................................19-20
   3.7 Transformation of Prepared E. coli and PUC 18..............................20-21
   3.8 Transformation of E. coli cells using PUC18 Adsorbed to Clay........21-23
   3.9 Plasmid Cut with Restriction Enzyme with EcoRI.........................23-24
   3.10 Phenol Extraction (Clay Mixed with PUC18)...................................25-26
   3.11 Transformation of bound DNA and Cut with EcoRI......................26
   3.12 X-Ray Diffraction Measurement..................................................26-28

4. Results
   4.1 Agarose mini-gel of PUC18 Cut with PVUII....................................29-30
   4.2 PUC 18 Transformation....................................................................30-31
   4.3 Increasing Amount of PUC18 Mixed with Constant Clay Amount....31-33
   4.4 Constant Amount of PUC18 Mixed with Increasing Clay Amount.....33-34
   4.5 Agarose mini-gel of PUC18 Mixed with Clay and Cut with EcoRI....35-36
   4.6 Transformation of Plasmid Mixed with Clay and Cut with EcoRI.....36-37
   4.7 Results of X-Ray Diffraction of Clay..............................................37-39
5. Discussion........................................................................................................40-42

6. Conclusion.........................................................................................................42-43

References...........................................................................................................44-46

Appendix A...........................................................................................................47

Appendix B...........................................................................................................48-49

Appendix C...........................................................................................................50-51

Appendix D...........................................................................................................52-53

Appendix E...........................................................................................................54
1. Introduction

Soils play an important role in the development of bacterial communities and their evolution. The relationship between soils and microbes depends on the components of soil and the type of microorganisms that are present. This work aims to examine the process by which soil minerals (montmorillonites) interact with DNA to facilitate the transfer of genetic information and how these minerals may protect DNA from degradation. Experiments were conducted using *Escherichia coli* (*E. coli*, hereafter) and the smectite clay, montmorillonite to determine the degree to which clay minerals protect DNA from degradation when exposed to natural enzyme activity.

Soils are aggregates of solid particles including organic and inorganic matter. The primary minerals are sand, silt and clay which account for 45% of soil inorganic matter. The inorganic materials provide the nutrients that plants need. The organic matter, which accounts for a small percentage of soil volume, consists of organic compounds derived from the remains of plants and animals as they decay.

Clay minerals are the primary mineral components of soils. Smectites are often referred to as “swelling clays”. Hydration leads to expansion of smectite clay, increasing the surface area and facilitating adhesion and reaction with molecules in the environment. Smectite is ubiquitous in soils and it is often associated with microorganisms such as bacteria, viruses and fungi that inhabit it. It is known that it plays an important role to the existence and activity of these microorganisms hence its specific effect upon them is often studied by biologists, ecologists and earth scientists. For example, it has been found that smectite has the ability to catalyze the
formation of biofilms, which are microecosystems comprised of populations of bacteria that can thrive attached to a surface (Alimova et al., 2007). In fact, Alimova et al. (2007) showed that smectite clay promoted biofilm formation which in turn supported the growth of three different types of bacteria (E. coli, Staphylococcus aureus, and Bacillus subtilis) under nutrient deficient. The interaction suggests that the abundance of bacterial biofilms in soils is directly related to the interaction between clay and biomolecules to create favorable growth conditions in the natural environment.

The relationship between smectites and organisms also extends to the degree to which biomolecules that contain genetic information may stably adhere to clay. Bacteria, like all organisms, have the ability to replicate and transfer their genetic material (DNA) to offspring. During reproductive processes, and as a result of radiation, viruses and anthropogenic factors errors may occur in the DNA code resulting in mutations that may increase or reduce a species ability to reproduce. The survival of organisms in the wild depends on the extent to which genetic information available in the environment in the form of DNA or RNA can be assimilated and the conditions that enable for the genetic information to be preserved.

In this work, determination of the degree to which a model soil (clay) can protect DNA from degradation and, in fact, serve as a repository and vehicle for DNA transfer to bacteria is tested via the following hypotheses: (1) Smectite clay acts as a DNA reservoir or delivery vehicle for bacteria; (2) smectite clay montmorillonite reduces the ability for a restriction enzyme to linearize the DNA and degrade it, (3) the DNA is expected to be attached to the clay by adsorption to the clay edges and faces as well as DNA fragments incorporated into the interlayer. The hypotheses will be tested by employing the following methods: transformation of E. coli, EcoRI restriction endonuclease cut, and X-ray diffraction.
2. **Background**

*Clay provenance and application*

Bentonites are thick deposits and source rocks for smectite clays. They form from the weathering of volcanic ash and its components vary depending on the type of alteration that has taken place (Christidis and Huff, 2009). Alteration is caused by interaction with meteoric and groundwater. However, bentonites can also form as a result of hydrothermal alteration of igneous rocks. As the parent material breaks down due to freezing, cooling, acid rain and other factors, the surface area increases and mineral soils containing clay minerals are formed. Some of the remaining particles become colloidal clay, that cannot be dissolved and recrystallize into tiny aluminosilicate platelets. Bentonites have multiple industrial uses where each application depends on the properties of a particular deposit.

Clays play an important role in soil microbiology, molecular and civil engineering and in environmental applications. Smectite is the main component of bentonites and are products of the weathering of mafic silicates. Montmorillonite (found in Montmorillon, France in 1788) is a type of smectite and it is known as clay minerals that compose smectites (Güven, 2009). Bentonites are named after Fort Benton, Wyoming, where large deposits are found due to weathering of tuffs and ash from the Yellowstone eruptions, and have been used as a healing agent in Mesopotamia around 2500 B.C and in ancient Egypt. In ancient Greece, bentonites were used as detergent to clean clothes (Elsenhour and Brown, 2009). Today they are used for a wide range of industrial applications including manufacture of cosmetics, pharmaceutical, and
contamination management. Additionally, bentonites have been used as environmental sealants as they are able to absorb water and fill boreholes, for paper making, and for metal casting such as street drains and manhole covers. In nuclear power plants, bentonites are used as barriers to isolate radioactive wastes in order to protect the environment and as in liners for managing hazardous waste, industrial spills etc (Gates et al. 2009).

Smectite structure

Clays can be classified by the ratio of tetrahedral to octahedral layers. Kaolinite, dickite, halloysite and nacrite for example, consist of one tetrahedral sheet joined with one octahedral sheet (i.e, 1:1). Smectite, pyrophyllite, chlorite, illite, and vermiculite for example contain one octahedral and two tetrahedral sheets (i.e, 2:1). Smectites are subdivided into dioctahedral and trioctahedral mineral systems (Brigatti et al, 2006 and Moore and Reynolds, 1997). Trioctahedral smectites have a ratio of 1:2 meaning that all three octahedral sites around each hydroxyl are filled whereas in dioctahedral system only two of the three available octahedral sites around each hydroxyl need to be occupied resulting in a 1:3 ratio. The formulas that represent the two groups are the following (Güven, 2009):

**Dioctahedral group formula:**

\[
[(M^{3+}_{2-y} M^{2+}_y) (Si^{4+} x M^{3+} x) O_{10} (OH)_2]^{(x+y)} E^{n+}_{(x+y)/n} \cdot nH_2O
\]

**Trioctahedral group formula:**

\[
[(M^{2+}_{3-y-z} M^{3+}_y M^{1+}_z) (Si^{4+} x M^{3+} x) O_{10} (OH)_2]^{(x+y+z)} E^{n+}_{(x+y+z)/n} \cdot nH_2O
\]

*E*\textsuperscript{n+} in the formulas above represents the exchangeable interlayer cation and M\textsuperscript{3+}, M\textsuperscript{2+} and M\textsuperscript{1+} represent the tetrahedral and octahedral coordinated cations. Table 1 below shows the smectite types of both groups with their appropriate name and charge distribution of cations in the different sheets (Güven, 2009).
Table 1 shows the mineral names and various cation distributions of the sheets of the two groups (figure from Güven, 2009).

A dioctahedral smectite, montmorillonite, was used throughout this research. It consists of one octahedral alumina sheet that is fused between two tetrahedral sheets with a variable basal (d_{001}) spacing of 9.6-21 Angstroms (Figure 1).
Figure 1: Smectite 2:1 layer structure (figure from Coulombe, 2007) where the blue represent the tetrahedral sheets, the purple represent the octahedral sheets and the space in between represents the interlayer.

In montmorillonites, each tetrahedron shares oxygen ions at three corners with three other tetrahedra in the tetrahedral sheet. The fourth is directed upward and when assembled they form a hexagonal sheet. The dominant cation in this sheet is Si$^{4+}$ that can be substituted by Al$^{3+}$ and Fe$^{3+}$. The octahedral sheet consists of two planes of closed pack oxygen ions. In between these planes cations exist that occupy the sheet. When the centers of these oxygen ions are connected they form an octahedron. The primary cations found on this sheet are Al$^{3+}$, Mg$^{2+}$, Fe$^{2+}$ or Fe$^{3+}$. Since the dimension of the tetrahedral sheet is greater than that of the octahedral there must be some adjustments in order to be able to fit one another and avoid the change in the hexagonal symmetry. These adjustments can occur in either the tetrahedral or the octahedral sheet and involve three mechanisms: the tetrahedra tilting or rotation and the difference in thickness of the
tetrahedral sheet. To change the thickness, the angle of the tetrahedron needs to be changed, thus when the angle increases the dimension of the sheet decreases. That can be also permitted by the movement of the anions around the vacant sites. To be specific when the anions move away from one another and towards the occupied sites the length of the edge of the octahedral increases from 2.7 Å to approximately 3.4 Å. The anions of the upper and lower planes move closer together and decrease the shared edges. The result of these movements is that the upper face rotates counterclockwise and it is pulled downward whereas the lower face rotates clockwise and it is pulled upward resulting in an ideal thickness of approximately 2.14 Å (Moore and Reynolds, 1997).

The sheet layers that are formed are about 1 nm thick and 200-1000 nm wide which qualifies clays as nanomaterials. When it comes to stacking the layers smectites undergo turbostatic disorder, hence they do not have a specific orientation. Stacked platelets or tactoids form due to the low layer charge, allowing a minimum concentration of the hydrated cation into the interlayer. This leads to weak attraction between the layers and interlayer cations leading to the development of large distances across the interlayer. This is what makes smectites swell when they come in contact with water as they become hydrated, resulting in a volume increase.

*Electrostatic charge in smectite*

There are two types of charges that are associated with clays minerals: the permanent charge is pH independent due to isomorphic substitution where every sixth aluminum cation is replaced by a magnesium cation and the variable charge that depends on pH (Eslinger and Pevear, 1988; Moore and Reynolds, 1997). The variable charge of clays is associated with the zero point charge (ZPC), defined as the pH (usually > 7) at which the clay can undergo anionic
or cationic exchange to neutralize the permanent negative charge. As a member of the
dioctahedral group, montmorillonite is known to have positively charged edges and negatively
charged surfaces with a resulting predominant permanent charge that is negative due to
isomorphic substitution. The charge is neutralized by cations that are found in the interlayer such
as K⁺, Mg⁺, Ca²⁺. As the octahedral and tetrahedral sheets are stacked together weak bonds are
produced that allow water and other molecules to enter the interlayer and cause expansion as
they force the layers apart whereas the interlayer allows cation exchange.

The ability to swell and exchange cations is also a result of the large surface area of
montmorillonite (~800 m²/gr), to yield a high cation exchange capacity (CEC), or how many
charged sites are available in the mineral. The CEC depends on the substitution of divalent ions
such as Mg²⁺ with Al³⁺ in the octahedral sheet and the presence of trivalent ions in the tetrahedral
sheet. The CEC of montmorillonite depends on the interlayer charge and varies with pH. CEC
increases when the pH is >6 which leads to the increase of metal-binding.

A double layer is formed when the negatively charged surface area is neutralized by
positively charged cations. In other words, when the negatively charged clays are suspended in a
solution, the cations are attracted to the clays to achieve neutrality. The exchangeable cations
diffuse from high concentration areas to low concentrations areas resulting in a diffuse layer with
a positive charge. This produces a zone around the clay with a variable thickness and decrease of
cation concentration as the distance from the clay increases. The variable thickness of the double
layer affects whether species can bind with the clay particles by Van der Waals forces or whether
they will stay in suspension as isolated entities (Moore and Reynolds, 1997). The double layer
may affect the adhesion of negatively charged bacteria onto clay as it acts as an energy barrier.
*(Genetic) Transformation in soil systems*

*Escherichia coli* or *E. coli* bacteria, discovered by Theodor Escherich, is a gram-negative enteric organism that exists in the intestines of animals and humans and can also be found in soil and water. *E. coli* is one of the most studied prokaryotic organisms having been described and mapped more than any other microorganism. The ubiquitous use of *E. coli* is related to the relative ease of growing the organism in the lab and the wealth of prior study of its structural and genetic makeup. This can be done because they allow genetic crosses to take place meaning the breeding that results in the offspring that caries genetic material from both parents. One thousand and nine hundred genes of have been managed to be mapped (Brock et al., 1994) (Figure 2). This makes it an ideal subject for use in biotechnology and genetic engineering.

![Figure 2: Genetic map of *E. coli* showing the gene map (figure downloaded from the web: http://bit.ly/VWCPVT).](http://bit.ly/VWCPVT)
For this research a non-pathogenic strain of *E. coli* was used, named XL-1 Blue with the genotype *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZΔM15 Tn10 (Tetr)]. This strain is resistant to tetracycline (Agilent Technologies Protocol).

Transformation is the process that permits the cell wall of the bacteria to become permeable to DNA, thus genetically altering the cells. In order for this to occur, the bacteria must be “competent”, meaning that cells must be in the state to pick up DNA. Once inside the cell the DNA can be recombined or degraded. Transformation, in other words, is a system by which genetic information is transferred between bacteria and results in their genetic change (Brock et al., 1994). This particular method by itself is not one of the most important methods in transferring genetic information in a natural environment like soil. That is because the DNA would require to be unprotected by a cell wall which can result in its quick degradation. Although many researchers support that this method is not a reliable one to transfer genetic information, Khanna and Stotzky (1992) demonstrated that when DNA interacts with the soil it is protected and transformation is successful due to clay particles in the soil that serve as a substrate for both bacteria and DNA. Therefore, when clay minerals are present in soil transformation can be an important tool in the alteration of the cells.

There are two types of DNA commonly used in transformation which are the chromosomal and the plasmid DNA. Plasmids in general are small circular DNA molecules that are often found in bacteria. Usually, they are used as cloning vectors and they are able to replicate. The genes in plasmids code for proteins that host organisms may lack and they also have the ability to break down antibiotics that may prevent the bacteria from growing. The plasmid employed in this study, PUC18, contains a gene called beta-lactamase which breaks down ampicillin and it is commonly referred to as the “selectable marker”. In other words once
this plasmid is introduced into the bacteria cells, they can grow the bacteria to become ampicillin resistant (Brock et al., 1994). In order to make *E. coli* ampicillin resistant, PUC18 was introduced into the cells by the transformation method.

**Clays and microorganisms**

Interactions between various microorganisms and clay minerals have been reported in relatively few scientific investigations for approaching different targets (see Table 2 for examples). Most experiments reported in the literature explore how the microorganisms behave in relation to different clay minerals and in what way the latter affects them. Adsorption of DNA to clays has been the interest of several studies since the phosphate ions in the backbone of DNA give it an overall negative charge, for example, same as clays. The factors influencing the adsorption and binding kinetics seem to be the type of clay and cations present, as well as the pH. Investigations suggested that the lower the pH the higher the adsorption (Khanna and Stotzky, 1992; Cai et al., 2006). In addition, in order for the bacteria’s DNA to overcome the diffuse double layer’s energy barrier and bind on the mineral, cation bridging must occur. The importance of divalent cations in the promotion of DNA adsorption to clays and sand has been reported by several studies (Cai et al., 2008; Cai et al., 2006; Saeki and Kunito, 2010; Nguyen et al., 2010; Romanowski et al., 1991). Since magnesium can be absorbed by both soil and organic matter it can act as a cation bridge for adhesion of negatively charged clay and DNA as it neutralizes the charges (Stotzky and Chenu, 2002). Additionally, several investigations have utilized montmorillonite and kaolinite clays in the exploration of adhesion. It has been suggested that montmorillonite provides higher adsorption percentage than kaolinite due to an increase in its surface area as well as due to its higher CEC value compared to that of kaolinite (Franchi et al., 1999).
Table 2: Examples of experiments involving clay minerals and microorganisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Clay</th>
<th>Experiment</th>
<th>Method/Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Montmorillonite</td>
<td>Chromosomal DNA, plasmid DNA, protection against EcoRI</td>
<td>Transformation</td>
<td>Gallori et al., 1994</td>
</tr>
<tr>
<td><em>Agrobacterium radiobacter</em></td>
<td>1:1, 2:1 clays</td>
<td>Bacterial growth</td>
<td>pH</td>
<td>Stotzky, 1966</td>
</tr>
<tr>
<td><em>Histoplasma capsulatum</em></td>
<td>Montmorillonite, Kaolinite</td>
<td>Respiration</td>
<td>Various concentrations of clays</td>
<td>Lavie and Stotzky, 1986</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Montmorillonite and kaolinite</td>
<td>Bacteriophage PBS1, UV radiation</td>
<td>Transduction</td>
<td>Vettori et al., 2000</td>
</tr>
<tr>
<td>Enzyme catalase</td>
<td>Montmorillonite</td>
<td>Adsorption and enzymatic activity</td>
<td>X-ray diffraction</td>
<td>Calamai et al., 2000</td>
</tr>
<tr>
<td><em>Reovirus type 3</em></td>
<td>Montmorillonite and kaolinite</td>
<td>Adsorption</td>
<td>Estuarine and distilled water adsorption</td>
<td>Stotzky and Lipson, 1983</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Montmorillonite and kaolinite</td>
<td>Chromosomal and plasmid DNA</td>
<td>X-ray, SEM, TEM, Transformation</td>
<td>Khanna et al., 1998</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Montmorillonite and kaolinite</td>
<td>Bacteriophage P1</td>
<td>Transduction</td>
<td>Zeph et al., 1988</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Montmorillonite</td>
<td>Chromosomal DNA, protection against DNase</td>
<td>Transformation</td>
<td>Khanna and Stotzky, 1992</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Montmorillonite and kaolinite</td>
<td>Chromosomal DNA</td>
<td>Transformation</td>
<td>Lotareva and Prozorov, 2000</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Montmorillonite, humic acids</td>
<td>Chromosomal DNA, protection against DNase</td>
<td>Transformation</td>
<td>Crecchio et al., 2005</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Montmorillonite and kaolinite</td>
<td>Pure and dirty DNA</td>
<td>Transformation</td>
<td>Pietramellara et al., 2007</td>
</tr>
</tbody>
</table>
According to Stotzky and Chenu (2002) one end of DNA binds to the edges of clay whereas the other end is free to attach on the competent cell. The adsorption can take place on both the interlayer and the edges of the mineral. Gallori et al. (1994) transformed the strains BD170 and BD1572 of *Bacillus subtilis* by DNA bound on montmorillonite and they found that the DNA was capable of transforming competent cells for up to 15 days in non sterile soil. In this same study, a restriction enzyme was also utilized to check if the DNA would degrade differently when bound to montmorillonite. The clay mineral protected DNA from linearization and degradation due to the restriction enzyme, and therefore, the DNA was able to transform to some extent.

In another investigation by Stotzky (1966) *Agrobacterium radiobacter* was suspended with 1:1 and 2:1 clay minerals at various pH conditions to determine whether the clay could buffer pH and permit bacterial growth. Montmorillonite was able to maintain a neutral pH (~7) longer than other clay minerals whereas kaolinite was not able to maintain pH after incubation.

The interaction between clay minerals and other microorganisms, e.g., fungi and viruses has also been studied. Lavie and Stotzky (1986) explored the interaction between *Histoplasma capsulatum*, a soil borne pathogenic fungus that causes histoplasmosis in humans, and montmorillonite and kaolinite to observe the effect of clay on fungus respiration and investigate the attachment between fungi and clay particles. By varying the concentration of clay they found that montmorillonite in low concentrations successfully reduced the respiration of the *histoplasma*. Even though the adhesion mechanisms require further investigation, they suggested that adhesion happened between the clay particles and the outer layer of the fungi and it was due to hydrogen bonding.
In addition, Vettori et al. (2000) investigated the role of clay minerals in protecting bacteriophage, PBS1, from UV radiation, which may damage phage DNA. Instead of transformation, they employed the transduction method which allows the transfer of genetic material from one bacterium to another by virus infection. They utilized strains of *Bacillus subtilis* susceptible to the phage and mixed these samples with montmorillonite and kaolinite. After exposing the samples to UV radiation for different time intervals it was found that montmorillonite had greater transduction efficiency than kaolinite but both clays protected the phage from UV radiation.

As evident from the summary of literature in the section above, few scientists have explored the interactions between clays and microorganisms. However, the relationship between *E. coli* and the plasmid PUC18 in the presence of montmorillonite has not been examined. In this work, the intent is to determine if clay acts as a vehicle to transfer genetic material to bacteria, and if it protects DNA from degradation caused by endonuclease action. Endonucleases can be found in soil and have the ability to degrade free DNA. Various studies have shown that DNA on the other hand, may be protected from such action if it is bound onto clays (Khanna and Stotzky, 1992; Gallori et al., 1994; Crecchio et al., 2005). In addition, it has been shown that once DNA binds on clays it is still able to transform and therefore clays may assist in the gene transfer (Figure 3). The success of this process in natural ecosystems is limited by the fact that soils are non-sterile environments and for that reason the probability that transformation occurs is small albeit possible. For this reason a model system was designed in this study to determine the feasibility and the role of clays on transformation in the presence of restriction enzymes. Since the genetic change is associated with the evolution of microorganisms, clay might play a significant role.
Some of the DNA binds on clays and gets protected from nucleases.

Figure 3: Model of transformation, natural and artificial and interaction with clay.
3. Materials and Methods

3.1 Bacteria, Plasmids and Restriction Enzymes

The non-pathogenic strain of *E. coli* used was XL-1 Blue with the genotype *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZΔM15 Tn10 (Tetr)]* as mentioned previously, purchased from Agilent Technologies of California (USA). Since many experiments would take place competent bacteria were grown from stock (XL-1 Blue). The kit included the plasmid PUC18 which is a vector, and in this study it was used as a DNA reagent that contains a gene for antibiotic resistance to ampicillin in *E. coli*. It contains also an enzyme called beta-galactosidase or *lacZ* (Figure 4). The last enzyme consists of a polylinker region where various restriction endonuclease enzymes are found. Plasmid PUC18 had to be purified in order to have a larger volume of it. For the experiments performed for this research only the prepared bacteria and plasmid were used.

Restriction enzymes, which are found naturally in bacteria, have made genetic engineering possible since they are able to cut DNA in smaller fragments and bind specific sequences. Two restriction enzymes were used: PVUII and EcoRI. PVUII was utilized in order to confirm the isolation of the plasmid.

In this work, EcoRI was used to show the bounded DNA’s resistance to the degradation by EcoRI. It allows digestion to occur and it creates a single cut on the plasmid in order to linearize it. In general that happens in order to permit the plasmid to recombine with foreign DNA which has undergone the same endonuclease action. Once the plasmid is cut, the DNA is presented by smaller fragments.

3.2 Preparation of Competent *E. coli* Cells

One litter of 2XYT media was prepared according to Fred M. Ausubel et al. (1994) (Appendix A) as well as 0.1 M of MgCl₂ and CaCl₂ respectively. One XL-1 Blue tube was streaked on an LB plate which was incubated at 37 °C overnight. One colony was then placed in a tube containing 10 ml of 2XYT medium, which was then transferred in the water bath with incubation at 37 degrees °C at 100 rpm overnight. The content of the tube was transferred into a flask containing 400 ml 2XYT and placed into the water bath (35 °C, 100 rpm) with incubation for two and a half hours. The mixture was then centrifuged in sterile centrifuge bottles at 5000 rpm for 10 minutes. It was re-suspended in 200 ml 0.1 M MgCl₂ and kept on ice for 10 minutes and then re-suspended in 200 ml of 0.1 M CaCl₂. It was then centrifuged at 5000 rpm for 10 minutes. It was re-suspended with 20 ml of 0.1 M CaCl₂ plus 14% glycerol and kept on ice for 20 minutes. 900 micro liters of the mixture were transferred to sterile eppendorf tubes and stored at -80 °C.
3.3 Clay Preparation

In order for the adhesion to occur smectite was prepared with MgCl₂ salt. Once the solution was saturated with the salt the magnesium was dissociated from chloride and it became Mg²⁺. The last managed to act as a bridge between the bacteria and clay as it succeed to neutralize the negative charge of the mineral in order to attract the negatively charged DNA molecules.

A sodium montmorillonite (commercial name: Accofloc 350; American Colloid Company) was prepared for this work using standard clay handling procedures described below. Its chemical formula is (Na,Ca)_{0.33}(Al_{1.67}Mg_{0.33})Si_{4}O_{10}(OH)_{2}.nH₂O. The Accofloc was purified to remove small amounts of crystalline silica, plagioclase, calcite and gypsum. It has a particle size of 200 mesh (74 microns) and is used in wastewater management to bind contaminants. Increasing the size of the floc can lead to higher treatment efficiency (AMCOL Paper Technologies).

Two grams of Accofloc 350 were mixed with 200 ml of sodium hypochlorite (Clorox bleach) to remove organics in a 100 ml tube. The tube was transferred into a cylinder placed on a hot plate containing water that had reached 69 °C. The clay was sonicated for 15 minutes. The mixture was transferred to a sterile centrifuge bottle and Milli-Q water was used to transfer the residue. The suspension was centrifuged at 13000 rpm for 20 minutes. The supernatant was removed completely and the pellet was re-suspended with Milli-Q water and centrifuged at 10000 rpm for 20 minutes for several times to remove the bleach. Then it was re-suspended in 0.1 M MgCl₂ to allow cation exchange overnight and centrifuged again at 13000 rpm for 30 minutes. It was then rinsed with distilled water and the supernatant was completely removed. This procedure was repeated several times. The mixture was transferred to a glass bottle in which
it was autoclaved twice. The concentration of the clay was calculated by weighing an empty petri dish and then adding 5 ml of clay amount and weighing again once the sample was dry, 3 days later. The concentration of the prepared clay was found to be 3.87 µg/µl.

3.4 Purification of Plasmid PUC18

The materials needed for the purification of the plasmid PUC18 were purchased from QIAGEN in a kit. The kit contained the protocol Bench Protocol: QIAGEN Plasmid Mini, Midi, and Maxi Kits. Some actions had to be performed before proceeding to the actual method. A detailed step by step description is included in Appendix B. The concentration of the plasmid was then calculated by using Nanodrop with a 1:10 dilution with a ratio 260/280 and was found to be 0.011 µg/µl. The real concentration of the plasmid was 0.11 µg/µl.

3.5 Preparation of Agarose Mini-gel

Different mixtures of PUC18 plasmid mixed with clay and/or cut with restriction enzymes were placed on Agarose mini-gels of 0.8% solution. The restriction enzyme PVUII (section 3.6) was used to confirm the isolation of PUC18. In addition, the restriction enzyme EcoRI was used to detect the degradation degree of the plasmid when the clay was or was not present (section 3.9). A step by step description is included in Appendix D.

3.6 Preparation of Plasmid Cut with Restriction Enzyme PVUII

PVUII Cut:

In order to ensure that the plasmid obtained from the purification method performed in the lab was PUC18, we had to perform a cut of it with the restriction enzyme PVUII that is well known. PVUII was purchased from BioLabs Inc of New England. This endonuclease enzyme can cut the plasmid and create smaller fragments. Once the circular plasmid PUC18 was cut, it
was placed in an agarose mini-gel of 0.8% solution (Appendix D) where according to the bands that would appear we would be able to ensure that it was the desirable plasmid that was isolated.

In order to cut the plasmid, one new and sterile eppendorf tube for each sample obtained from the purification method was used, where 2.5 µl of buffer 10X was added to each of them. 16.5 µl of distilled water was added to each tube as well as 1 µl of PVUII to all tubes. 5 µl of each of the tubes obtained from the purification method were added to the corresponding just prepared tube. The mixtures were then incubated for two hours at 37 °C water bath and stored at -20 °C freezer. The following day the mixtures shown in the table 3 below were positioned on the 0.8% agarose gel:

Table 3: Introduction of mixtures to agarose mini-gel wells

<table>
<thead>
<tr>
<th>Well</th>
<th>Sample</th>
<th>Sample Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>Ladder</td>
<td>2 µL DNA ladder</td>
</tr>
<tr>
<td>2nd</td>
<td>Uncut sample #1</td>
<td>1 µL PUC18 + 2 µL dye + 7 µL H2O</td>
</tr>
<tr>
<td>3rd</td>
<td>Uncut sample #2a</td>
<td>1 µL PUC18 + 2 µL dye + 7 µL H2O</td>
</tr>
<tr>
<td>4th</td>
<td>Uncut sample #2b</td>
<td>1 µL PUC18 + 2 µL dye + 7 µL H2O</td>
</tr>
<tr>
<td>5th</td>
<td>Uncut sample #3</td>
<td>1 µL PUC18 + 2 µL dye + 7 µL H2O</td>
</tr>
<tr>
<td>6th</td>
<td>Cut sample #1</td>
<td>8 µL mixture (cut PUC18) + 2 µL dye</td>
</tr>
<tr>
<td>7th</td>
<td>Cut sample #2a</td>
<td>8 µL mixture (cut PUC18) + 2 µL dye</td>
</tr>
<tr>
<td>8th</td>
<td>Cut sample #2b</td>
<td>8 µL mixture (cut PUC18) + 2 µL dye</td>
</tr>
<tr>
<td>9th</td>
<td>Cut sample #3</td>
<td>8 µL mixture (cut PUC18) + 2 µL dye</td>
</tr>
<tr>
<td>10th</td>
<td>------</td>
<td>------</td>
</tr>
</tbody>
</table>

3.7 Transformation of Prepared E. coli and PUC 18

In order to check the transformation efficiency of the prepared plasmid, the transformation method was employed and different masses of DNA were used. As stated before the concentration of the prepared plasmid was found to be 0.11 µg/µl which after a 1:10 series dilution yielded a concentration of 11 ng/µl. The masses of the plasmid calculated for this experiment were 1.1 ng, 11 ng, 110 ng and 220 ng according to the volumes used which were 0.1
µl, 1 µl, 10 µl and 20 µl respectively. The procedure was followed as described in the kit protocol with minor adjustments where five 15 ml tubes were pre-chilled instead of two and instead of adding 1 µl of PUC18 the above volumes were used. One eppendorf tube containing competent E. coli cells was placed on ice for 40 minutes. SOC Medium was preheated in 42 ºC bath. 100 µl of E. coli were transferred to each 15 ml tube while it was kept on ice. 1.7 µl of B-Mercaptoethanol that was included in the kit, was added to each tube to increase the transformation efficiency. The tubes were swirled gently every 2 minutes for a total of 10 minutes. The above volumes of PUC18 were then added to each of the tubes. The fifth tube was the control (no DNA added). The mixtures were swirled gently once and incubated on ice for 30 minutes. They were heat-shocked in the water bath of 42 ºC for exactly 45 seconds. They were placed on ice and incubated again for 2 minutes. 900 µl of SOC media was then added to both tubes and incubated at 35 ºC and at 100 rpm for 1 hour. Then they were plated on LB-Ampicillin plates following a series of 1:10000 dilutions. The remaining mixture of the actual tubes was then centrifuged and most of the supernatant was removed. The approximately 100 µl of the specimen was also plated. The plates were then incubated in 37 ºC overnight and colonies that were grown were measured.

3.8 Transformation of E. coli Using Prepared PUC18 Adsorbed to Clay

This experiment was performed twice in a different time: for the first experiment a constant amount of clay with a mass of 38700 ng (or 3.87 × 10^4 ng) was used and mixed with increasing amounts of PUC18 plasmid. For the second experiment a constant amount of PUC18 (11 ng) was used and mixed with increasing amounts of clay.
1st experiment:

PUC18 that was obtained after the plasmid purification was diluted in a series of 1:1000. The following mixtures (Table 4) were prepared in eppendorf tubes. There were only three control samples, #1, #2, and #3, which contained unbound DNA and six samples that contained DNA adsorbed to clay (samples #4, #5 #6, #7, #8, #9). The controls (#1, #2, #3) were chosen to be three, and not six instead as the bound DNA samples, because if inhibition would take place it was expected to occur in samples #4, #5 and #6, which contained the least amount of DNA that could be used (with the clay amount being constant and high).

Table 4: Preparation of mixtures (mass of clay and DNA used). For this experiment clay amount was kept constant. Samples #1, #2 and #3 with the notation * in the sample column represent the controls (unbound DNA).

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Mass of PUC18 (ng)</th>
<th>Mass of Clay (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>0.11</td>
<td>0</td>
</tr>
<tr>
<td>2*</td>
<td>0.99</td>
<td>0</td>
</tr>
<tr>
<td>3*</td>
<td>9.9</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.11</td>
<td>3.87 ×10⁴</td>
</tr>
<tr>
<td>5</td>
<td>0.99</td>
<td>3.87 ×10⁴</td>
</tr>
<tr>
<td>6</td>
<td>9.9</td>
<td>3.87 ×10⁴</td>
</tr>
<tr>
<td>7</td>
<td>99</td>
<td>3.87 ×10⁴</td>
</tr>
<tr>
<td>8</td>
<td>1.1 ×10⁴</td>
<td>3.87 ×10⁴</td>
</tr>
<tr>
<td>9</td>
<td>1.001 ×10⁴</td>
<td>3.87 ×10⁴</td>
</tr>
</tbody>
</table>

2nd experiment:

PUC18 that was obtained from the purification method was diluted following a 1:100 series with a resulting mass of 11 ng which was used for all samples. The clay was also diluted following a 1:10 series for sample #1 while all other samples were undiluted. The following mixtures (Table 5) were then prepared in eppendorf tubes.
Table 5: Preparation of mixtures (mass of clay and DNA used). For this experiment DNA amount was kept constant. Sample #6 with the notation * in the sample column represents the control (unbound DNA).

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Mass of Clay (ng)</th>
<th>Mass of PUC18 (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$3.87 \times 10^4$</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>$1.935 \times 10^5$</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>$3.87 \times 10^5$</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>$1.935 \times 10^6$</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>$3.87 \times 10^6$</td>
<td>11</td>
</tr>
<tr>
<td>6*</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

All of the above tubes were incubated at room temperature for 2 hours. The procedure followed the exact same path as described under section 3.7. Minor adjustments had to be made again where instead of pre-chilling 5 tubes of 15 ml the number was adjusted to the tubes described in the tables above plus one additional for each experiment that contained no DNA and/or clay. In addition, the volume of the tubes prepared replaced the amount of PUC18 suggested in the protocol.

For the first experiment we plated the mixtures by following a series of 1:10000 dilutions on LB-Ampicillin plates. For the second experiment we used a series of 1:100 dilutions of the mixtures. A detailed step by step method is included in Appendix C.

3.9 Preparation of Plasmid Cut with Restriction Enzyme EcoRI

EcoRI Cut:

EcoRI enzyme was also purchased from BioLabs Inc. of New England and it is an endonuclease enzyme well known, mapped and isolated from an *E. coli* strain. It is used to
restrict plasmids and permit cloning. EcoRI as well as other enzymes are used to linearize the circular plasmids and cut it at one single site. In this case, the enzyme was used to linearize PUC18 in order to prove that it would not allow any transformation to take place if it was just combined with DNA but in the same time when it was combined with DNA-clay the last would protect DNA and there would be some transformation. Stotzky et al. (1994) followed a similar procedure when he was binding clay with plasmids on *Bacillus subtilis* in non-sterile soil using transformation.

**Method:**

In order to perform the experiments the volumes of materials had to be calculated. Since the concentration of DNA was known (the desired volume of it was 20 µl and the mass of PUC18 was found to be 2.2 µl). The units of EcoRI used for this experiment were found to be 5.5 (unit information provided from the enzymes’ manual) which resulted in 0.3 µl. The volume of buffer 10X was adjusted from the total volume of the mixture (50 µl) which was found to be 5 µl.

One eppendorf tube was used and incubated at room temperature for 2 hours before the procedure which contained 20 µl of PUC18 and 20 µl of clay. Two hours later 0.3 µl of EcoRI, 5 µl buffer 10X, and 4.7 µl of distilled water were added in the tube. Another eppendorf tube was prepared which contained 0.3 µl of EcoRI, 5 µl buffer 10X, 20 µl of PUC18 and 24.7 µl of distilled water. Both tubes had a total volume of 50 µl. The procedure followed the same path as described in section 3.6, by using the above volumes instead and just before they were stored in the -20 °C freezer they had to be placed in the 65 °C water bath for 20 minutes to stop the reaction. The tubes were then stored in the -20 °C freezer.
3.10 Phenol Extraction (on bound DNA)

When the plasmid bound to clay was cut with EcoRI could not be placed directly on the agarose mini-gel to determine if it was the desirable one and if the plasmid was indeed linearized. The phenol extraction procedure was employed in order to extract DNA by separating it from any proteins acids and in our case also from the clay matrix and concentrate it in order to be positioned on the gel.

Method

For the procedure, 25 µl of the second tube prepared in the above method that contained the combined clay and plasmid which was cut with EcoRI were mixed with 25 µl of distilled water. 50 µl of this mix were combined with 50 µl of Phenol-Chloroform- Isoamyl alcohol mix. The mixture was shaken by Vortex and then it was centrifuges at 14,000 rpm for 4 minutes. The aqueous (upper) layer was removed carefully and repeatedly and transferred into a new sterile eppendorf tube. 2.5 of total volume (~150 µl) of cold absolute ethanol were added into that tube and it was kept in -20 °C for 2 hours. The specimen was then centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed and the pellet was dried by the air vacuum. 15 µl of distilled water were added to the dry pellet and it was gently re-suspended for several times. The tube was then stored at -20 °C. The following day an agarose mini gel of 0.8 % solution (Appendix D) was prepared and the following mixtures were introduced to it (Table 6):

Table 6: Introduction of mixtures to agarose mini-gel wells

<table>
<thead>
<tr>
<th>Well</th>
<th>Sample</th>
<th>Sample Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>Ladder</td>
<td>2 µL DNA ladder</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>Uncut sample</td>
<td>1 µL PUC18 + 2 µL dye + 7 µL H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td>4th</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>-----</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>5th</td>
<td>Cut PUC18</td>
<td>2 µL mixture + 2 µL dye + 6 µL H₂O</td>
</tr>
<tr>
<td>6th</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>7th</td>
<td>Cut PUC18+ Clay (after phenol extraction)</td>
<td>8 µL mixture + 2 µL dye</td>
</tr>
<tr>
<td>8th</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>9th</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>10th</td>
<td>-------</td>
<td>-------</td>
</tr>
</tbody>
</table>

3.11 Transformation of Plasmid Mixed with Clay and Cut with EcoRI

Method

The following tubes were prepared with a total volume of 20 microliters: the 1st tube contained PUC18 only and a series dilution of 1:100 was performed. Then 10 µl of the mixture were taken and combined with 10 µl of distilled water. The 2nd tube contained DNA bound to clay and EcoRI. A 1:100 series dilution was followed. 2.5 µl of mixture were taken and mixed with 17.5 µl of distilled water. The 3rd tube contained clay and PUC18. 10 µl of clay were used and combined with 10 µl of PUC18 (1:100 dilution). The 4th tube consisted of PUC18 and EcoRI diluted in 1:100 series. 2.5 µl of mixture were taken and were combined with 17.5 µl of distilled water. The tubes were transferred in 27 °C incubator with shaking for 2 hours. The transformation procedure followed the same route as described previously in section 3.7 (or Appendix C) in which the amount of PUC18 added was replaced with the volume of the above tubes. The series of the dilutions used for this experiment was 1:1000 that were plated on LB-Ampicillin plates.

3.12 X-ray Diffraction Measurement

In order to examine the effect of the interaction of clay minerals and microorganisms on mineral structure, X-ray diffraction method was utilized. X-ray diffraction is the most efficient method to identify and analyze clay particles whose crystal structure varies when it comes into
contact with water or other solutions. XRD enables identification of the mineral and its crystal structure, i.e., the geometrical distribution of the atoms contained in the crystal.

A crystal is a solid with long range order of atoms in three dimensional space with planes measured in angstroms and the angles between crystal axes measured in degrees to define the basic unit or unit cell. XRD uses a monochromatic beam of an appropriate wavelength which passes through the plane and allows the x-rays to be reflected by the atoms of the crystal. The family of planes is defined by 3 integers $h$, $k$ and $l$ that are known as Miller Indices and they are written in the form $(hkl)$. Each index represents a plane orthogonal to the directions $h$, $k$ and $l$. The signal or peak in an XRD scan is the result of constructive interference caused by the interaction between families of planes and reflected x-rays that are in phase. Bragg’s law states that $n\lambda = 2d \sin \theta$ where $d$ is the space between atomic planes or the inter-planar distance of the crystal, $\lambda$ (CuKα 1.5418 Å) is the wavelength meaning the angle between the beam and the atomic plane and finally $n$ is an integer number that defines the order of diffraction. Diffraction will only occur if Bragg’s law is satisfied. In single crystal diffraction, as a crystal is rotated its planes diffract x-rays and Bragg’s law is satisfied. In powder diffraction, crystallites are randomly oriented to achieve the same effect. In the case of a homogeneous clay mineral sample, an oriented slide where the 00$l$ planes are preferentially reflected is utilized for first order identification of mineral features (Moore and Reynolds, 1997).

The X-ray diffraction instrument consists of a generator, a sealed X-ray tube, a goniometer and a detector (Figure 5). The generator supplies the X-ray tube with high voltage and the sealed x-ray tube transfers energy to the atoms that results in the creation of vacancies when it bombards the electrons. The goniometer rotates the sample following Bragg’s law conditions and the detector permits the measurement of the intensity of the X-ray transmitted.
Smectites that have been saturated by a cation, in this case Mg$^{2+}$ have all interlayer vacancies filled leading to a uniform and stable expansion of the interlayer to bring $d_{001}$ to 14 Å.


Samples of clay and PUC18 were analyzed to determine if DNA adsorption affected clay structure. The first sample contained 200 µl of the prepared clay. The second sample consisted of 200 µl of clay mixed with 2 µl of the prepared PUC18. The third sample contained 200 µl of clay mixed with 1 µl of the prepared PUC18. Once they were mixed they were kept in room temperature for 2 hours. They were then centrifuged for 3 minutes at 13000 rpm and the supernatant was removed. They were re-suspended and each mixture was placed on a glass slide appropriate for the XRD instrument covered by another glass slide. The slits used were $\frac{1}{2}^\circ$, $\frac{1}{2}^\circ$ and a PM = $6.4^\circ$ anti-scattering slit. The time for the program to run was set to 6 minutes from $4^\circ$ to $32^\circ$. All degrees stated refer to degrees 2θ.
4. Results

4.1 Agarose Mini-gel of Plasmid PUC18 Cut with PVUII

The following samples were introduced to the agarose 0.8% mini-gel to detect if PUC18 was purified and isolated by cutting it with the restriction enzyme PVUII (Table 7).

Table 7: Samples of uncut PUC18 and samples of PUC18 cut with PVUII introduced in the various wells of the agarose mini-gel.

<table>
<thead>
<tr>
<th>Well #</th>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ladder</td>
</tr>
<tr>
<td>2</td>
<td>Uncut plasmid sample #1</td>
</tr>
<tr>
<td>3</td>
<td>Uncut plasmid sample #2a</td>
</tr>
<tr>
<td>4</td>
<td>Uncut plasmid sample #2b</td>
</tr>
<tr>
<td>5</td>
<td>Uncut plasmid sample #3</td>
</tr>
<tr>
<td>6</td>
<td>Cut plasmid sample #1 with PVUII</td>
</tr>
<tr>
<td>7</td>
<td>Cut plasmid sample #2a with PVUII</td>
</tr>
<tr>
<td>8</td>
<td>Cut plasmid sample #2b with PVUII</td>
</tr>
<tr>
<td>9</td>
<td>Cut plasmid sample #3</td>
</tr>
<tr>
<td>10</td>
<td>------------</td>
</tr>
</tbody>
</table>

Figure 6 below shows the resulting bands of above mixtures. The uncut PUC18 for all samples 1, 2a and 2b appeared in multiple bands. Sample 2b is the most highlighted compared to the other two because it seems to have the highest molecular weight compared to the other samples. PVUII cuts the plasmid twice so it creates three smaller fragments. The cut samples 1, 2a and 2b appeared in two bands (the third cannot be seen) as indicated in the figure. The program Gene Runner gave the fragments sizes which were 308 bp, 322 bp and 2056 bp and are shown in Figure 6. Fragment size 2056 is clearly exposed on the figure for all three DNA samples and it is located in the upper part of the gel. The other two fragments are further down and since they are very close to each other they cannot be distinguished. Since PVUII cuts PUC18 in these fragments according to its genetic map the resulting plasmid was indeed PUC18.
4.2 PUC18 Transformation

The purified plasmid prepared in the lab yielded the following results obtained from the transformation method: when the mass of PUC18 was 1.1 ng there were no transformed colonies for all diluted and undiluted samples. When 11 ng of plasmid were used, only the undiluted sample had 4 colonies which translate to 40 transformants/ml. The number of colonies increased even more as soon as the mass reached 110 ng, where the undiluted sample as well as the 1:10 dilution had >100 (TNC) transformed colonies. The 1:100 diluted sample yielded 68 colonies which translate to 68000 transformants/ml and the 1:1000 had 5 colonies which convert to 50000 transformants/ml. The mixture containing 220 ng had >100 (TNC) transformed colonies for 1:10 dilution. The 1:100 diluted mixture showed 135 counted colonies which translates to 135000 and the 1:1000 plate showed 8 colonies that
converts to 80000 transformants/ml. Table 8 shows the average of the transformants/ml of each mass and Figure 7 shows the increase of the transformation due to the different masses.

Table 8: Average of transformed colonies/ml of PUC18 resulted from different masses.

<table>
<thead>
<tr>
<th>PUC18 Mass (ng)</th>
<th>Transformants/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.1 \times 10^1$</td>
<td>$4 \times 10^1$</td>
</tr>
<tr>
<td>$1.1 \times 10^2$</td>
<td>$5.9 \times 10^3$</td>
</tr>
<tr>
<td>$2.2 \times 10^3$</td>
<td>$1.075 \times 10^5$</td>
</tr>
</tbody>
</table>

Figure 7: As the mass of PUC18 increased the transformants/ml kept rising.

4.3 Increasing Amount of PUC18 Mixed with Constant Clay Amount

In this experiment increasing amounts of DNA were mixed with a constant amount of clay which had a mass of $3.87 \times 10^4$ ng. The samples explained in Table 4 gave the following results: for the control #1 (0.11 ng) tube the undiluted mixture yielded 14 colonies. For the control sample #2 (0.99 ng) the undiluted mixture yielded 140 colonies and 1:10 dilution showed 6 colonies. The control sample #3 (9.9 ng), 1:10 and 1:100 diluted mixtures had 91 and 10 transformed colonies respectively. The undiluted sample had >100 (TNC) colonies. The combined DNA and clay sample #4 (0.11 ng) had 14 colonies for the undiluted mixture.
and the 1:10 dilution on plate showed only 2 colonies. Tube #5 (0.99 ng) of the combined specimen showed 150 colonies for the undiluted sample whereas the 1:10 dilution mixture showed 15. Tube #6 (9.9 ng) of the combined mixture showed >100 (TNC) colonies for the non-diluted sample, 72 colonies for the 1:10 fold and 8 colonies for the 1:100 dilution. Tube #7 (99 ng) had >100 (TNC) colonies for both the undiluted and 1:10 dilution mixture. In addition 87 colonies appeared in the 1:100 dilution plate and 7 in the 1:1000 plate. Tube #8 (1100 ng) had >100 (TNC) for the undiluted, 1:10 and 1:100 dilution. The same tube showed 71 colonies and 11 colonies for 1:1000 and 1:10000 diluted mixtures respectively. At last tube #9 (10010 ng) had >100 (TNC) for the undiluted, 1:10 and 1:100 diluted samples. Then 1:1000 and 1:10000 dilutions showed 186 and 23 transformed colonies respectively. The transformed colonies were then translated to transformants/ml and their average was taken (Table 9) in order to represent it in the graph below (Figure 8) which shows the increase in transformation efficiency of all DNA absorbed to clay samples. It also shows that inhibition did not occur in the three samples of bound DNA (#4, #5 and #6) in relation to the controls as there was no decrease of transformed colonies (Table 9). The red line represents the increase of the controls (DNA samples) and the blue line represents the increase of the combined samples (DNA bound on clay samples).

Table 9: It shows the averages of transformed colonies per ml that kept increasing as the DNA mass kept rising while the clay amount was kept constant. Samples #1, #2 and #3 with the notation * in the sample column represent the controls (only PUC18).

<table>
<thead>
<tr>
<th>Samples #</th>
<th>PUC18 Mass (ng)</th>
<th>Clay Mass (ng)</th>
<th>Transformants/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>0.11</td>
<td>0</td>
<td>$1.2 \times 10^2$</td>
</tr>
<tr>
<td>2*</td>
<td>0.99</td>
<td>0</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td>3*</td>
<td>9.9</td>
<td>0</td>
<td>$5.05 \times 10^3$</td>
</tr>
<tr>
<td>4</td>
<td>0.11</td>
<td>$3.87 \times 10^4$</td>
<td>$1.7 \times 10^2$</td>
</tr>
<tr>
<td>5</td>
<td>0.99</td>
<td>$3.87 \times 10^4$</td>
<td>$1.5 \times 10^4$</td>
</tr>
<tr>
<td>Sample</td>
<td>PUC18 Mass (ng)</td>
<td>DNA Mass (ng)</td>
<td>Transformants/ml</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>---------------</td>
<td>------------------</td>
</tr>
<tr>
<td>6</td>
<td>9.9</td>
<td>$3.87 \times 10^4$</td>
<td>$6.55 \times 10^3$</td>
</tr>
<tr>
<td>7</td>
<td>$9.9 \times 10^4$</td>
<td>$3.87 \times 10^4$</td>
<td>$7.85 \times 10^4$</td>
</tr>
<tr>
<td>8</td>
<td>$1.1 \times 10^4$</td>
<td>$3.87 \times 10^4$</td>
<td>$9.05 \times 10^5$</td>
</tr>
<tr>
<td>9</td>
<td>$1.001 \times 10^4$</td>
<td>$3.87 \times 10^4$</td>
<td>$2.08 \times 10^6$</td>
</tr>
</tbody>
</table>

Figure 8: It shows the transformans/ml as the mass of PUC18 was increasing while the clay amount was kept constant. As PUC18 mass was increasing the transformants/ml kept rising also in all bound DNA samples (even in the ones with smaller DNA amounts) indicating that inhibition did not occur meanwhile the prepared PUC18-clay mixture was still able to transform.

4.4 Constant Amount of PUC18 Mixed with Increasing Clay Amount

For this experiment all undiluted samples explained in Table 5 and plated showed >100 transformed colonies. 67 colonies and 6 colonies appeared in sample #1 as it was diluted in 1:10 and 1:100 respectively. Sample #2 had 76 and 4 colonies for the dilutions mentioned last. Sample #3 had 70 and 9 transformed colonies for the same dilutions. Sample #4 showed 44 and 6 colonies following the same dilutions as above. Sample #5 showed 31 transformed colonies for the 1:10 dilution plate and 6 colonies for the 1:100 dilution. At last sample #6 (control-only DNA) had 74 and transformed colonies as it was diluted to 1:10 and 6 for the 1:100 dilution plate. These were translated to transformants/ml and their averages were taken (Table 10).
9 represents the decrease as clay amount increased (bound DNA samples represented in blue) compared to the control DNA sample (red line).

Table 10: It shows the averages of transformants/ml of the various samples that contained increasing amount of clay and constant amount of DNA. Sample #6 with the notation * in the sample column represents the control (PUC18 only).

<table>
<thead>
<tr>
<th>Samples #</th>
<th>Clay Mass (ng)</th>
<th>PUC18 Mass (ng)</th>
<th>Transformants/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$3.87 \times 10^4$</td>
<td>11</td>
<td>$6.35 \times 10^5$</td>
</tr>
<tr>
<td>2</td>
<td>$1.935 \times 10^5$</td>
<td>11</td>
<td>$5.8 \times 10^5$</td>
</tr>
<tr>
<td>3</td>
<td>$3.87 \times 10^5$</td>
<td>11</td>
<td>$8 \times 10^5$</td>
</tr>
<tr>
<td>4</td>
<td>$1.935 \times 10^6$</td>
<td>11</td>
<td>$5.2 \times 10^5$</td>
</tr>
<tr>
<td>5</td>
<td>$3.87 \times 10^6$</td>
<td>11</td>
<td>$4.55 \times 10^5$</td>
</tr>
<tr>
<td>6*</td>
<td>0</td>
<td>11</td>
<td>$6.7 \times 10^5$</td>
</tr>
</tbody>
</table>

Figure 9: Increasing the clay mass while keeping the amount of DNA constant resulted in a decrease of transformed colonies/ml. Some inhibition started occurring in samples #4 and #5, as shown in the graph where the clay amount was higher, even though the result is not conclusive.
4.5 Agarose mini-gel of Plasmid PUC18 Mixed with Clay and Cut with EcoRI

Table 11 shows the mixtures introduced to the gel in order to see if PUC18 was cut and linearized with EcoRI. In addition, this experiment and method showed if clay protected DNA from the endonuclease. It also showed if the plasmid was purified of clay particles and proteins and concentrated since the phenol extraction was employed.

Table 11: Distribution of mixtures to agarose mini-gel wells

<table>
<thead>
<tr>
<th>Well #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well # 1</td>
<td>Ladder</td>
</tr>
<tr>
<td>Well # 2</td>
<td>Ladder</td>
</tr>
<tr>
<td>Well # 3</td>
<td>Uncut plasmid PUC18</td>
</tr>
<tr>
<td>Well # 4</td>
<td>----------</td>
</tr>
<tr>
<td>Well # 5</td>
<td>Cut plasmid PUC18 with EcoRI</td>
</tr>
<tr>
<td>Well # 6</td>
<td>----------</td>
</tr>
<tr>
<td>Well # 7</td>
<td>Clay sample after phenol extraction</td>
</tr>
<tr>
<td>Well # 8</td>
<td>----------</td>
</tr>
<tr>
<td>Well # 9</td>
<td>----------</td>
</tr>
<tr>
<td>Well # 10</td>
<td>----------</td>
</tr>
</tbody>
</table>

Figure 10 below shows the selected plasmid sample 1 as uncut in well #3. There are two distinguished bands shown on the gel. Well #5 shows the plasmid after it was cut with EcoRI. Gene Runner gave a fragment size of 2686 bp. There was only one band represented because it cuts it only once. Well #7 represents the DNA bounded to clay and cut with EcoRI. Phenol extraction that was performed to that sample allowed the bands to appear and move on the gel and created three fragments. The result was that the plasmid was indeed linearized by the enzyme (well 5) meaning it would not be able to transform, and clay protected it from further endonuclease action (well 7), therefore it would be able to transform.
4.6 Results of Transformation of Plasmid Mixed with Clay and Cut with EcoRI

DNA control sample #1 yielded >100 transformed colonies (TNC) from the undiluted sample. The same result was repeated for tube #3 (bound DNA). Tube #2 (where EcoRI cut was performed on bound DNA) had 115 colonies instead. Tube #1 had 56 colonies and 7 colonies appearing from dilution mixtures 1:10 and 1:100 respectively. Tube #2 had 0 transformed colonies for the exact same dilutions. Tube #3 had 44 and 3 colonies respectively for the dilutions last mentioned while tube #4 (EcoRI cut on plasmid PUC18) had 0 transformed colonies for all undiluted, 1:10 and 1:100 dilutions. The colonies were then translated to transformants/ml and the averages of each mixture resulting colonies were calculated. These values appear in table 12 below. Finally Figure 11 shows the result of these mixtures which increases or decreases and even reaches 0 according to the contents each tube (for the mixtures refer to the method’s section “Transformation of Plasmid Mixed with Clay and Cut with EcoRI”).
Table 12: Averages of transformants/ml after EcoRI cut on plasmid (sample #4) and on bound DNA (tube 2). Tube 2 supported that clay protected DNA from EcoRI action as it transformed to some extent whereas tube 4 showed 0 transformants when EcoRI cut DNA and clay was not present. The averages of transformants/ml of the control (sample #1) and of the bound DNA-no EcoRI (sample 3) are also shown in this table.

<table>
<thead>
<tr>
<th>Samples #</th>
<th>Sample Content</th>
<th>Transformants/ml (undiluted)</th>
<th>Transformants/ml (1:10 dilution)</th>
<th>Transformants/ml (1:100 dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>TNC</td>
<td>$5.6 \times 10^3$</td>
<td>$7 \times 10^5$</td>
</tr>
<tr>
<td>2</td>
<td>EcoRI on bound DNA</td>
<td>1150</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Bound DNA</td>
<td>TNC</td>
<td>$4.4 \times 10^3$</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>4</td>
<td>EcoRI on PUC18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 11: Diagram shows the differences in results of DNA control, EcoRI cut of bounded DNA to clay, bound DNA and EcoRI cut on plasmid. The second bar from the left shows the undiluted sample’s transformants/ml when EcoRI cut was performed on bound DNA and the fourth bar shows the 0 transformation when EcoRI cut was performed just on DNA.

4.7 Results of X-ray Diffraction

Figure 12 a, b and c below shows the XRD spectra of a) clay sample (control), b) clay mixed with 1 µl DNA and c) clay mixed with 2 µl DNA. The 001 is at ~6.5° 2θ, the 002 peak is broadened and it is not visible in the scan. However, the 003 and 004 at ~18.5 and ~25.5 degrees 2θ are well defined in the scan of the control sample (Figure 12 a). The other two spectra on figures 12 b) and c) seem to follow almost the same path as the clay sample. Their 001 peak has
almost the same degrees 2θ whereas their 002 peak is not visible on the scan. However their 003 and 004 peaks seem to be wider and broader as the DNA amount increases. A significant change though is not observed between the spectra suggesting that the absorption did not occur in the interlayer.

Figure 12 a) Clay sample
Figure 12 b) Clay sample mixed with 1 µl DNA

Figure 12 c) Clay mixed with 2 µl DNA
5. Discussion

From the results obtained in the above section it was shown that the transformation was successful in all experiments. In the case of increasing mass of PUC18 while the clay mass was constant the transformation efficiency was higher than when the clay mass was increased and the mass of the plasmid was kept constant. In the first experiment, where the constant was the clay amount and the variable was the DNA amount, there was not inhibition occurring in the bound DNA samples. Furthermore, according to the controls, there was no inhibition occurring even in the three samples that consisted of the smallest amount of DNA that could be used, which were bound to high and constant amount of clay. That resulted in the suggestion that the amount of clay used was not sufficient to inhibit all DNA molecules, and therefore to result in the decrease of the transformation efficiency. That led to the second experiment where higher amounts of clay were used. In that experiment, where the amount of clay was increased while the volume of DNA was kept constant resulted in a decrease in transformed colonies, thus showing that little, if any, inhibition occurred. Although increasing the clay concentration resulted in a lower efficiency of transformation when compared to the DNA control mixture, suggesting that inhibition occurred, this result is not conclusive. The lessened effect of the restriction enzyme on DNA when clay was present suggests that the interaction between clay and DNA protects DNA from endonuclease cut. However, insufficient data was collected to ascertain whether DNA binds on clay. In the future, in order for us to confirm the data obtained, and further investigate the binding of clay and DNA, the experiment should be reproduced in triplicate. One sample of bound DNA would be used which would be divided in two tubes. The first sample would be centrifuged and the supernatant would be used during transformation whereas the second would be used as is during transformation. A third sample would be used as the control which would
not contain clay. The same mass of DNA and similarly increasing masses of clay would be used. Inasmuch, the same dilutions would be used, the mean values (averages) of the transformants/ml of these three same experiments would be taken and a statistical analysis will be employed. The statistical analysis should include the standard deviation and error bars on the graph representing the results obtained. In addition, a Student’s t-test would be applicable in order to compare the means of the samples. An alternative test would be to use the same statistical analysis from one single sample containing the same masses as mentioned above which would be spread on the plates using same dilutions in triplicates. Even though further investigation needs to be carried out to be precise, the data obtained, implied but did not prove that clay can be used as a vehicle to transfer genetic information between bacteria in the environment where both of these components are found and which may lead to the genetic alteration of these microorganisms.

DNA bound to clay degraded less when restriction enzyme EcoRI was added to the complexes. When the transformation of cells where unbound DNA was cut with EcoRI there was no transformation. In contrast, when the enzyme was added to the DNA-clay complex, the transformation effectiveness increased from 0 to 115 colonies. Therefore, clay was still able to maintain circular DNA morphology even though the enzyme’s effect is to linearize the molecules. Clay-protected DNA was still able to transform competent cells and also prevented degradation that would have taken place had the mineral not been present. This result is in agreement with Stotzky’s et al. (1994) investigation of the transformation of Bacillus subtilis by bound DNA and how the latter was also protected from nuclease action.

The results obtained from the x-ray diffraction indicate no significant difference in basal spacing. The 003 and 004 peaks appeared slightly broader in samples where DNA was bound to the clay compared to the clay control. The XRD spectra suggest that the DNA particles were not
introduced and attached to the interlayer. Furthermore, the neutrality on the faces of the clay due to the pretreatment of MgCl₂ may have reduced the ability for DNA to adsorb to these surfaces. However, the edges of the clay may serve as a repository for DNA fragments. The binding or non-binding mechanisms of DNA to clay seem to be the product of Coulomb’s law of attraction where opposite sign charges attract each other and charges of the same sign repel each other. XRD does not show an increase in basal spacing indicating that DNA did not significantly intercalate clays, even though it was capable of transforming competent cells, and agrees with the studies investigated by Khanna et al. (1998), and Franchi et al. (1999).

6. Conclusion

The transformation of *E. coli* absorbed to clay throughout this research was successful. It was shown that by increasing the amount of clay to an adjusted constant amount of DNA, the transformation could still take place and resulted in the decrease of the transformants even though there was minimum effectiveness. Conversely keeping the clay amount constant and increasing the amount of DNA resulted in the increase of transformants. However, the transformation was still successful. Additionally, it was also demonstrated that EcoRI did not allow any transformation to take place when mixed with the unbound plasmid, as expected, but when combined with DNA bounded to clay transformed colonies were observed. The results obtained did not conclusively support the hypothesis that (1) smectite serves as a pool for DNA to transform *E. coli*, hence it transfers genetic information and the role of clay in this study needs to be further investigated. The data obtained supported hypothesis (2), that clay protects DNA from degradation due to the endonuclease action of EcoRI. Even though DNA was probably absorbed to the clay edges and not on the faces, hypothesis (3), that DNA fragments could be incorporated into the interlayer, was not supported through this research. There was no
significant difference in the basal spacing of the bound DNA compared to the clay sample as obtained from the X-ray diffraction method that could indicate the change of the interlayer due to DNA adsorption. The question of how DNA gets absorbed into the clays still remains unanswered. Inasmuch if DNA is physical adsorbed by the clay surface or if it penetrates into the interlayer and thus forming organic material.

Future investigations to determine the interaction between clays and DNA should include various types of clays such as 2:1 and 1:1 groups as well as diverse strains of a variety of bacteria microorganisms. They should also include different types of restriction endonuclease to compare these future results to those of the present work in order to verify or contradict them. Multiple studies have been conducted for the knowledge of these mechanisms but they are still many questions regarding the nature of the adhesion, which may depend on the type of bacteria and clay that are present and, therefore, need clarification. These concepts can play an important role in the future to understand the evolution of bacteria. In conclusion, they are a key to microbial ecology and the development of genetically altered bacteria in the environment.
References


Appendix A

Media

2XYT Media

Per liter:

1. Add 16g Bacto Tryptone.
2. Add 10g Bacto Yeast Extract.
3. Add 5g NaCl.
4. Adjust to 1L with distilled H₂O.
5. Sterilize by autoclaving.

LB Solution and Plates

Per liter:

1. Add 10 g Bacto Tryptone.
2. Add 5 g Bacto Yeast.
3. Add 5g NaCl.
4. Autoclave to sterilize

For LB plates add to the above mixture 15 g Bacto Agar.

In order to make LB-Ampicillin plates 1 ml of Ampicillin was added to the above mixture.

SOB/ SOC Media

The recipes for these media can be found in the Agilent Technologies manual that was included in the kit or electronically.
Appendix B

PUC18 Purification

Materials

LB-Ampicillin Medium
Transformed competent *E. coli* (on plates)
QIAGEN Plasmid Midi Kit
50 ml tubes
Sterile centrifuge tubes

Method

A) Bacterial culture, harvest and lysis

- Add 5 ml LB-Ampicillin Medium into a 50 ml tube.
- Pick up one single colony and transfer into the above tube.
- Incubate it in the 35 °C water bath overnight.
- Add 20 ml of LB-Ampicillin Medium into a flask. Pour the mixture of the tube in the flask and incubate it at 27 °C overnight.
- Follow the Bench Protocol for Midi Preparation.
- Re-suspend the pellet in 4ml of Buffer P1.
- Add 4ml of Buffer P2 and mix by inverting the tube a few times.
• Incubate at room temperature for 5 minutes.
• Add 4ml of Buffer P3 and mix by inverting the tube a few times.
• Incubate on ice for 15 minutes.

B) Bacterial lysate clearing

• Centrifuge at 20000 rpm for 30 minutes at 4 °C. Re-centrifuge the supernatant at 20000 rpm for 15 minutes at 4 °C.

C) Bind, wash and elute plasmid DNA on QIAGEN-tip

• Equilibrate a QIAGEN-tip 100 by applying 4 ml of Buffer QBT and allow column to empty by gravity flow.
• Apply supernatant to the QIAGEN-tip and allow it to enter the resin by gravity flow.
• Wash the QIAGEN-tip with 10 ml of Buffer QC (repeat twice: 2x10 ml). Allow Buffer QC to move through the QIAGEN-tip by gravity flow.
• Elute DNA with 5 ml Buffer QF into clean 15 ml vessel.

D) Precipitate, wash, and redissolve plasmid DNA

• Precipitate DNA by adding 3.5 ml room-temperature isopropanol to the eluted DNA and mix. Centrifuge at 15000 rpm for 30 minutes at 4 °C. Carefully decant supernatant.
• Wash DNA pellet with 2 ml room-temperature 70% ethanol and centrifuge at 15000 rpm for 10 minutes. Carefully decant supernatant.
• Air-dry pellet for 5-10 minutes by using the air-vacuum and re-dissolve DNA in 200 microliters of distilled water.
Appendix C

Transformation of *E. coli* cells Using Plasmid PUC18 Adsorbed to Clay

Materials

Competent *E. coli* cells
15 ml tubes
Eppendorf tubes
PUC 18
B-Mercaptoethanol
SOC Medium
Lb- Ampicillin plates

Method

- Pre-chill as many 15ml tubes as needed for each experiment and label them according to the above eppendorf tubes.
- Pre-chill one more 15 ml tube used as a control (no DNA/clay) for each experiment.
- Place *E. coli* on ice for 30-45 minutes.
- Preheat SOC Medium to 42 °C waterbath.
- Once the *E. coli* is melted put 100 microliters to each of the 15 ml tube without removing from ice.
• Add 1.7 microliters of B-Mercaptoethanol to each tube.

• Swirl the tubes gently and incubate on ice by swirling every 2 minutes.

• Add mixture of each eppendorf tube prepared to the corresponding 15 ml tube. Swirl the tubes gently.

• Incubate them on ice for 30 minutes.

• Heat shock tubes in 42 °C water bath for exactly 45 seconds.

• Incubate the tubes on ice for 2 minutes.

• Add 900 microliters of SOC Medium to all tubes and incubate them at 35 °C and at 100 rpm for 1 hour.

• For the first experiment plate them following a series of 1:10000 dilutions on LB-Ampicillin plates. For the second experiment use a series of 1:100 dilutions.

• Incubate them overnight at 37 °C.

• Count colonies.
Appendix D

Agarose Mini-Gel 0.8%

Materials:

50 ml TBE Buffer 10X
Plastic Cylinders
Mini gel Box and Comb
1.5 microliters EtBr (Ethilium Bromide)
450 ml distilled water

Method:

- Pour 50 ml of TBE Buffer 10X into plastic cylinder
- Pour 450 ml of distilled water into the same plastic cylinder
- Pour 30 ml of that to mix into 125ml flask.
- Weigh 0.3 grams of Agarose LMP and pour it into the flask.
- Place flask on Hot Plate Stirrer to boil
- Once it boils remove from the Stirrer and let it cool for 5-10 minutes.
- Add 1.5 microliters of EtBr and mix.
- Pour mixture into tray of gel box and burst bubbles with plastic tip of pipette.
- Place comb on the top side of side of the tray and let the mixture solidify.
• Rotate tray until black electrode is on the top left corner and the comb is on the same side.

• Pour the rest mixture of TBE Buffer and distilled water in the center of gel until it is filled and it completely covers it.

• Remove comb by pulling it straight upwards.

• Start the dilutions and pour it into each of the wells of the gel.

• Cover it with its plastic lid by attaching it to the appropriate electrodes and let it run for one hour at 90 volts.
Appendix E

A few plates shown below with transformed colonies (number of transformed colonies written with blue marker on top) throughout the various experiments. The blue spots on the plates represent the transformed colonies measured.