NATURAL TRANSFORMATION AND GROWTH INHIBITION OF AZOTOBACTER VINDELANDII IN THE PRESENCE OF ADSORBED OXYTETRACYCLINE

BY

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THESIS

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ABSTRACT

Antimicrobial resistance genes and antimicrobials are introduced to agricultural soil through land application of manure. Both can persist in the environment if adsorbed to soil grains, allowing potential for increased antimicrobial resistance in the soil microbial community. Oxytetracycline (OTC) is a commonly used antimicrobial in large-scale livestock production systems that can persist in the environment. To better understand how adsorbed antimicrobials could affect antimicrobial resistance, the mechanism of OTC adsorption to a clay soil was investigated using sorption isotherm experiments and modeling, Fourier transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD). Sorption data showed a good fit with a cation-exchange capacity sorption model. FTIR results revealed oxytetracycline sorption to this clay soil by electrostatic interactions between the protonated dimethylamino group of oxytetracycline and the negative charge on the surface of the soil. XRD results revealed that OTC adsorbed to the surface of non-expandable illites and significantly influenced how water molecules interact with expandable clay minerals. In addition, the influence of oxytetracycline adsorbed to sterile soil on the natural transformation and inhibition of the soil bacterium *Azotobacter vinelandii* was investigated. The transformation frequency by both plasmid and chromosomal DNA was not significantly affected by the addition of adsorbed OTC, showing that the presence of OTC does not hinder gene transfer. A higher concentration of adsorbed OTC compared to dissolved OTC was required to inhibit growth, which may select bacteria in the soil community.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

Antimicrobials are becoming more prevalent in the soil environment due to land application of manure and sewage solids to fertilize agricultural land *(1)*. Antimicrobials are designed to kill or inhibit growth of microbes; as a result, the possible effects of released antimicrobials on the soil microbial community are becoming more of a concern. A change in the microbial structure, an increase in antibiotic resistance, and a change in soil ecological functions are possible outcomes. The microbial community structure can change due to the selective antibiotic effects and can interfere with the interactions between various species *(2, 3)*.

Oxytetracycline (OTC) is a common antibiotic used in large-scale livestock farms and has been found in the environment in varying concentrations. In swine lagoons, OTC with other tetracyclines has been detected in the range of 25-410 μg mL^{-1} *(4)*. After application of manure to soil, OTC has been found at 1691 μg kg^{-1} *(5, 6)*. Compared to other antimicrobials, OTC has slow degradation probably due to its strong sorption to soil *(7, 8)*. Very little OTC has been detected in leachate, showing slow mobility of OTC in the environment *(9, 10)*.

OTC shows strong sorption to soils with clay contents ranging from 5.2-16.9%, with no significant desorption observed *(9-11)*. In general, the amount of OTC adsorbed to clays decreases with an increase in pH *(12-15)*, and the dominating mechanism at higher pH is hydrophobic interactions *(13)*. Montmorillonite and kaolinite are common clay minerals and differ in structure. For all pH values, OTC adsorbs more to montmorillonite than kaolinite. The most important mechanism for OTC sorption to montmorillonite is cation exchange *(12)*. Since calcium is preferred by the clay in acidic conditions, it could take up sites and not allow oxytetracycline to adsorb. In alkaline conditions, calcium enhanced sorption of OTC to

montmorillonite, because of calcium cation bridging to the negatively charged OTC *(12)*. Even though kaolinite has a lower cation exchange capacity, tetracycline adsorbs on the external surfaces by cation exchange rather than forming metal complexes *(16)*.

Besides clay minerals, organic matter and iron oxides play an important role on OTC sorption. An increased amount of dissolved organic matter and an increase in soil pH was found to decrease the sorption of OTC to clay soils, suggesting hydrophobic interactions dominate at higher pH *(13)*. In addition, metal bridging allows increased adsorption of OTC to organic matter *(17)*. Tetracycline sorption coefficients increased with pH for iron oxides, with a maximum at pH 8 and with surface complexation being the dominant mechanism. Digests with sodium citrate-sodium dithionite showed oxide components to be more important than clay interactions *(18)*. Overall, tetracycline sorption showed little influence from soil organic matter content; the most influential mechanisms were cation exchange capacity and iron oxide content *(19)*.

Antimicrobials have multiple effects on the soil microbial community including changing the structure, increasing resistance and altering the soil's ecological functions. Antimicrobial resistance can be directly measured by the abundance of resistance genes. An increase in the abundance of these genes with time has shown an increase of antibiotic resistance *(20)*. More information is needed to study the implications of an increase in resistance genes. Antimicrobials in soil have also affected ecological functions such as nitrification and denitrification processes *(2, 21)* and microbial activities and enzymes *(2, 22)*.

Few studies have focused on the biological effects of adsorbed antimicrobials. Inhibition of soil bacteria occurs with OTC present in sandy soils, but little information is known of inhibition in clay-rich soils *(1)*. Tetracycline adsorbed to soil was still biologically active against *Salmonella* and *Escherichia coli*, although these experiments were performed with

concentrations above those typically found in the environment *(23)*. For oxytetracycline and sulfapyridine, a correlation has been established between strong adsorption and a smaller and delayed antibiotic effect, presumably since a stronger adsorption means a lower bioavailability of antibiotic *(3)*.

Antimicrobials that adsorb to clay particles are still biologically active and may influence the prevalence of antibiotic resistant bacteria *(24)*. However, it is unclear as to how much of the effect in previous studies is due to adsorbed antimicrobials. More information is also needed to see if adsorbed antimicrobials have an influence on gene transfer.

The objectives of this research are: (1) to investigate the mechanism of oxytetracycline adsorption to a clay soil and, (2) to determine whether adsorbed oxytetracycline influences transformation and inhibition of a soil bacterium, *Azotobacter vinelandii*. Batch adsorption isotherms and Fourier transform infrared spectroscopy (FTIR) results were complemented with clay characterization by X-ray diffraction (XRD) and soil composition analysis. OTC adsorbed at varying concentrations to sterile soil was added to competent *A. vinelandii* and plasmid DNA, and to *A. vinelandii* and growth media to examine possible effects of resistance and inhibition, respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

20mM morpholinepropanesulfonic acid (MOPS) with 1mM $CaCl_2$ transformation buffer was prepared with nanopure water and adjusted to pH 7.0-7.2 with NaOH. Oxytetracycline hydrochloride (OTC) (MP Biomedical, Solon, OH) was dissolved daily in nanopure water. Solutions were filtered with 0.22-μm-pore-size nylon membranes before use.

Analytical Methods. An Agilent Series 1200 HPLC instrument (Santa Clara, CA) with an Agilent Zorbax Eclipse XDB-C18 column, 150 x 4.6 mm, particle size 5 μm, was used for separation. Detection was conducted at 355 nm. The optimized mobile phase consisted of 77% nanopure water, 23% acetonitrile (ACN) HPLC grade, and 0.1% formic acid (pH 2.9). The flow rate was 0.7 ml min^{-1}.

2.2 SOIL CHARACTERIZATION AND PREPARATION

Soil from the South Farms of the University of Illinois Urbana-Champaign campus was used. The soil was classified as Drummer silty clay loam *(25)* and has no exposure to manure with tetracycline residues. Previous soil samples from this location contained 3.4 ng of oxytetracycline per g of soil (dry weight) *(26)*. Soil wash analytes were analyzed by ICP-OES (PerkinElmer Optima 2000DV ICP-OES). BET surface area was determined using the Accelerated Surface Area and Porosimetry System (Micrometircs, Norcross GA). Cation exchange capacity (CEC) was determined by the compulsive exchange method *(27)*. Carbon was analyzed with a UIC Coulometrics Model 5014 CO_2 Coulometer equipped with a CM 5200 Autosampler Furnace. Samples were analyzed for total carbon and for organic carbon by treatment with sulfurous acid until the end of CO_2 evolution. Iron and aluminum oxide content

were determined by extraction using both the acid-ammonium oxalate (AAO) and dithionite-citrate-bicarbonate (DCB) methods *(28)*. Extracts from these methods were analyzed by ICP-OES. To sterilize the soil, a 5 g aliquot of soil was microwaved for 5 minutes, autoclaved for 60 minutes, and dried overnight at 90°C.

2.3 XRD ANALYSIS

Mineralogical analyses of the soil samples were performed at the Illinois State Geological Survey (ISGS) by methods developed previously *(29-31)*. Samples were analyzed after three different processes: (1) air drying, (2) treating with ethylene glycol (EG) for 24 hours, and (3) heating the slide at 350°C for one hour. These same treatments were applied to samples with OTC adsorbed to see how the OTC impacts the soil samples. 25 mL of the separated clay suspension (0.02 g clay mL^{-1}) was added to a 50 mL centrifuge tube. 2 mL of 2 mg mL^{-1} OTC solution and 15 mL of DI water was added and equilibrated by rotation overnight. Two desorption washes were completed (as described in the following section) before analyzing with XRD.

To prepare XRD slides, approximately 20-30 g of untreated soil was allowed to soak with DI water overnight in a covered 100 ml beaker. The sample was then mixed and allowed to settle overnight. After settling, one third of the clear supernatant was carefully poured off and then refilled with DI water and two drops of sodium hexametaphospate dispersant. After mixing and settling for 15 min, the sample was pipetted onto a glass slide, which was allowed to dry overnight before being analyzed with a Scintag® XDS2000 machine. Step-scanned data was collected from 2° to 34° 2θ with a fixed time of 5 seconds per 0.05°2θ for each sample. All resulting traces were analyzed using the semi-quantitative data reduction software from Materials Data Inc. (MDI) known as Jade+®. Relative clay mineral contents were calculated by

comparing peak height ratios among expandable clays (smectite or mixed layer smectite), illite, kaolinite and chlorite. Qualitative analysis of trace overlays was conducted by comparing the diffractograms of the same sample after the oriented slide has undergone various treatments (i.e. heated, glycolated, or air dried).

2.4 OXYTETRACYCLINE ADSORPTION, DESORPTION, AND ISOTHERMS

125 mg of sterile soil (m_{soil}) was added to a 50 mL centrifuge tube. 0.25 mL of 1mg mL^{-1} OTC solution (M_T = 250 μg) and 50 mL of MOPS solution was added to the soil. The soil mixture was rotated at 2 rpm for 20-24 hours at room temperature. After the 20-24 hour rotation, the soil mixture was centrifuged at 50 *g* for 15 minutes. The supernatant was removed, filter sterilized, and measured with HPLC analysis. The aqueous equilibrium concentration (C_e) was determined from HPLC analysis using calibration curves. The adsorbed OTC concentration q_e was calculated by mass balance.

Desorption of oxytetracycline was also monitored. After removing the supernatant, 50 mL of MOPS solution was added to the soil. The soil mixture was again rotated at 2 rpm for 20-24 hours and centrifuged at 50 *g* for 15 minutes. The supernatant was removed, filter sterilized, and measured with HPLC analysis to determine the equilibrium concentration after one wash (C_1). This process was repeated again to ensure little OTC desorbed from the soil. The concentration of OTC that was still adsorbed to the soil after the first ($q_{e,1}$) and the second ($q_{e,2}$) washed was calculated by mass balance.

Isotherms were also obtained in the same way but varying the amount of OTC solution, ranging between 4.5 – 27 μg mL^{-1}. K_d (mL g^{-1}) values were calculated as the ratios of adsorbed concentration q_e (μg g^{-1}) and the aqueous phase concentration C_e (μg mL^{-1}). Control experiments confirmed no adsorption of OTC to the 50 mL centrifuge tube by HPLC analysis before and after

a one-day rotation. Isotherm data were fitted to Freundlich (equation 1) and Langmuir models (equation 2).

$q_e = K_f C_e^{\,n}$ (1) where q_e (mmol/kg) is the adsorbed concentration; C_e (mmol/L) is the equilibrium aqueous concentration; K_f is the Freundlich coefficient and n is the exponent;

$q'_e = \frac{QbC_e}{1+bC_e}$ (2) where q'_e (mmol/m^2) is the adsorbed concentration normalized by surface area; Q (mmol/kg) is the maximum adsorption capacity; and b (L/mmol) is the Langmuir coefficient

2.5 FTIR ANALYSIS

The oxytetracycline adsorption and desorption procedure was followed but modified to start with 2.5 mg of oxytetracycline to 125 mg of soil to increase the signal. 125 mg of soil without OTC adsorbed was also used for the background of the OTC-soil complex. Following centrifugation and supernatant removal, the OTC-soil complex and soil pellets were freeze-dried. Soil was dispersed in FTIR-grade potassium bromide (Sigma-Aldrich). FTIR measurements were recorded using a Nexus 670 with a DRIFTS accessory, scanning with a 4 cm^{-1} resolution and 256 scans. Background for the OTC spectra was KBr, while the background for the OTC-soil complex was the soil pellet dispersed in KBr. Spectra were analyzed and refined using SpecManager software from Advanced Chemistry Development (ACD Labs, Toronto Ontario).

2.6 BACTERIAL STRAINS, CULTURE MEDIA, AND GROWTH CONDITIONS

Plasmid DNA pBR325 and pDB17 were grown in *E. coli* strain DH5α cultured in LB media (10g Tryptone, 5 g yeast extract, 5 g NaCl, 1 mL 1N NaOH dissolved in 1 L of DI water) with carbenicillin at 50 μg mL^{-1} (pBR325 source DSMZ, pDB17 *(32)*). Wild type *Azotobacter vinelandii* strain DJ (source Dr. Dennis Dean) was used as recipient of plasmids pBR325 and pDB17. The strain was cultured in Burk's (B) medium *(33)* at 30°C and 170-rpm shaking. Burk's medium with 0.013 mol l^{-1} ammonium acetate (BN medium) was also used.

For chromosomal DNA transformations, DNA was purified from *A. vinelandii* DJ using standard procedures *(34)*. Strain DJ77 was used as the recipient. This strain has a 128-bp deletion in the *nifH* gene, preventing nitrogen fixation when molybdenum is present.

2.7 DNA PURIFICATION

Plasmid DNA was purified from *E. coli* cultures grown at 37°C for 16-18 hours following the QIAGEN Plasmid Maxi Kit protocol. Plasmid DNA was suspended in endotoxin-free water and stored at -20°C. The concentration of DNA was calculated with UV spectroscopy and quantified by agarose gel electrophoresis.

2.8 BACTERIA SEPARATION FROM SOIL

To remove bacteria from soil mixture, a soil wash protocol was developed. All of the methods are described below; the one that was used in subsequent experiments is given first. Since microscope detection was not necessary for the OTC work, the method that yielded the best recovery of cells was used. 5 sterile glass beads (5 mm in diameter) were added to soil mixture. Samples were vortexed for 1 minute and centrifuged at 5 *g* for 5 minutes. The supernatant (containing bacteria) was diluted and plated. This method has a recovery that ranges from 69 to 94% when compared to viable cell counts.

Several methods were attempted for optimum cell recovery and different uses (microscope detection and minimal soil contamination when using plate counts). A previous method developed for wood chips was slightly modified *(35)*. 2.5 g of soil and 5 5mm glass beads were added to 10 mL of sterile 1X PBS buffer (130 mM NaCl and 10 mM sodium phosphate ($Na_2HPO_4 \bullet 7H_20$), adjusted to pH 7.2) in a 50 mL centrifuge tube. This solution was vortexed for 1 minute and centrifuged at 750 *g* for 5 minutes. The supernatant was decanted into a fresh tube. This bacterial solution was concentrated and resuspended in 200 μL of MOPS

buffer. 5 μL of solution was spotted on a slide and stained with DAPI (4',6'-diamidino-2-phenylindole, 2 μg mL^{-1}) for 5 minutes. The slide was viewed under the DAPI filter set for cells and the FITC filter set for background fluorescence. This method yielded a low cell count and high background fluorescence.

To remove background fluorescence, the highest centrifuge setting (50 *g*) was used to pellet the soil particles. 5 μL of the suspension from the first centrifugation was spotted and stained on a slide. 5 μL of the resuspended bacterial pellet was also spotted and stained on a slide. A higher cell count was present in the suspension when compared to the pellet. Therefore, the bacteria were concentrated in the first centrifugation.

Spotting the bacteria on a microscope slide may not be the best way to detect bacteria. A 0.2 μm filter was used to see if the filter collects bacteria from the wash. The same wash protocol was followed, but after the solution was decanted, it was filtered through a 0.2 μm filter. The filter was stained with DAPI and viewed under the microscope under the DAPI and FITC filter set. A much higher cell count was seen on the filter, but it was difficult to focus on one focal plane since there was too much soil debris being caught in the 0.2 μm filter. Very high background fluorescence was present.

Different centrifuge settings were used to test whether or not soil debris could be removed before using the 0.2 μm filter. The centrifuge speed before filtering the solution was varied. Three different speeds were tested, 0 *g*, 5 *g*, and 50 *g* (corresponding to resting on bench, 500 rpm, 5000 rpm for 5 minutes). The 0 *g* setting resulted in high background fluorescence and a high cell count. The low speed (5 *g*) resulted in high background fluorescence and a lower cell count. The high speed (50 *g*) resulted in the lowest background fluorescence and the lowest cell count.

Sodium pyrophosphate (NaPPi) has been known to reduce background fluorescence by breaking up clay colloids *(36)*. 1% NaPPi (10X NaPPi solution consisted of 10 g of NaPPi dissolved in 100 mL deionized water) was added to the supernatant after all three centrifuge settings (0 *g*, 5 *g*, 50 *g*). The low speed seemed to give the best results with the NaPPi present with still low cell counts.

Another method attempted to remove background fluorescence was to filter out the debris before using the 0.2 μm filter. The supernatant from the middle centrifuge setting (5 *g*) was passed through an 8.0 μm filter. Different volumes of the filtrate were then collected on a 0.2 μm filter. 1 mL and 100 μL of the filtrate were used. The 0.2 μm filters were stained and viewed under the microscope. Low background fluorescence was present. The cell count was calculated and determined to be close to the value of what is recovered from soil in other literature.

The 8.0 μm filter may actually trap some bacteria. To check this, the 8.0 μm filter was stained with DAPI and viewed under the microscope. There was some bacteria seen, but only about 2% of the total amount recovered.

To ensure that this method (5 *g* centrifuge setting, 8.0 μm filter, and 0.2 μm filter) would yield the lowest background fluorescence and the highest cell count, NaPPi was also added to the filtrate from the 8.0 μm filter. This solution was then passed through the 0.2 μm filter. The NaPPi did not remove the remaining background fluorescence.

2.9 NATURAL TRANSFORMATION ASSAYS

Assays consisted of three things in a 1.5 mL centrifuge tube: 400 μL competent cells, 5 μL plasmid DNA, and 600 μL dissolved OTC solution or adsorbed OTC slurry (250 mg of soil total). For competence, wild type *A. vinelandii* DJ was grown on BN plates without

molybdenum at 30°C for two days, inoculated into BN liquid media, and grown at 30°C for 18 to 20 hours with shaking (170 rpm). The dissolved OTC solution was from either a measured solution (for higher OTC concentrations) or a supernatant from the OTC adsorption experiment. The adsorbed OTC slurry consisted of two centrifuge tubes of pelleted soil resuspended in residual supernatant. Transformation assays were rotated for 20-24 hours at 2 rpm at room temperature.

Dissolved transformation mixtures and supernatants from soil washes (described above) were then diluted with sterile phosphate buffer (0.16 M KH_2PO_4 and 0.51 M K_2HPO_4 dissolved in DI water) and spread onto plates. Dissolved transformation mixture was spread onto B media with carbenicillin at 20 μg mL^{-1} concentration and B plates. Soil wash supernatant was spread on B media with carbenicillin and B media with cycloheximide at 400 μg mL^{-1} concentration to remove fungal contamination. The plates were incubated at 30°C for 4 days. Transformation frequencies were calculated by dividing the number of transformants (colonies on B with carbenicillin plates) by the number of viable cells (colonies on B or B with cycloheximide plates) in the transformation mixture. Detection limits were found for each experiment by counting the negative control viable cells. Frequencies above detection limit were included.

Chromosomal DNA was also used in natural transformation experiments with OTC present. Dissolved and adsorbed natural transformation assays included the use of chromosomal DNA isolated from *A. vinelandii* strain DJ. Assays were plated on B media (transformants) and BN media (viable cells).

2.10 DEVELOPMENT OF RIF^R *A. VINELANDII* MUTANT

A strain derived from *A. vinelandii* DJ resistant to rifampin was developed. 200 μL *A.vinelandii* was added to BN media plate with 10μg mL^{-1} of rifampin to identify a spontaneous

mutant resistant to rifampin. After 5 days of growth at 30 °C, a colony was seen and streaked on another BN media plate with rifampin.

2.11 OXYTETRACYCLINE INHIBITION ASSAYS

A single colony of *A. vinelandii* DJ grown on a BN plate was inoculated into BN media and grown for 41.5 hours to ensure growth at the stationary phase. Two mixtures: OTC adsorbed to soil or OTC dissolved in MOPS buffer. For the OTC slurry, OTC adsorption and desorption methods were followed. At the end of the second wash, pelleted soil resuspended in residual supernatant from one centrifuge tube was diluted with pelleted soil without OTC to obtain a specific amount of oxytetracycline adsorbed. The final volume of slurry was 500 μl. For the other condition, OTC was also dissolved into 500 μl of MOPS buffer. For both experimental conditions, 6 ml of BN media and 50 μl of a starter culture was added to each OTC mixture in a 50 ml centrifuge tube. A positive control of MOPS buffer, BN media, and starter culture, but no OTC was used. Two negative controls were used: MOPS buffer and BN media; and slurry and BN media. All mixtures were incubated at 30°C for 26 hours with shaking at 170 rpm. Mixtures with soil slurry were washed, and the supernatant was diluted and spread on BN media with cycloheximide at 400 mg l^{-1}. Mixtures without soil slurry were diluted and spread on BN media.

2.12 STATISTICAL ANALYSIS

To analyze transformation frequencies and sorption values, Bartlett's test was used to test homogeneity of variances. Since no statistical difference was observed between sets of values, a two-sample t test was used to compare frequencies. A P value of 0.05 was considered significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 PHYSICAL AND CHEMICAL PROPERTIES OF SOUTH FARM SOIL

Characteristics of this soil that may influence adsorption include high organic carbon (3.0±0.10%) and cation exchange capacity (CEC) (14.4 cmol kg^{-1}). It is low in iron and aluminum oxides (DCB Fe: 1.46 mmol kg^{-1}, DCB Al: 0.61 mmol kg^{-1}) when compared to soils used in other studies on OTC adsorption *(18, 37)*. Additional soil characteristics are summarized in Table 1.

South Farm Soil Characteristics	
Surface Area (m^2/g)	4.51
Total C (%)	3.3±0.20
Organic C (%)	3.0±0.10
DCB Fe (mmol/kg)	1.46
DCB Al (mmol/kg)	0.61
AAO Fe (mmol/kg)	0.51
AAO Al (mmol/kg)	1.36
K_d (ml g^{-1})	1804 ±260.25
K_d (L m^{-2})	0.40 ±0.058

Table 1. South Farm soil characteristics.

Soil wash analytes	
C (ppm)	2.35
Si (ppm)	5.62
S (ppm)	7450
Na^{+} (ppm)	0.62
K^{+} (ppm)	1.63
Mg^{2+} (ppm)	0.93
Ca^{2+} (ppm)	1.72
Cl^{-} (ppm)	0.54

Table 1 (cont.). South Farm soil characteristics.

3.2 SORPTION ISOTHERMS FOR SOUTH FARM SOIL AND OTC PROPERTIES

To evaluate OTC sorption to the South Farm soil, sorption isotherms were conducted and are shown in Figure 1. The isotherms were fit to both Freundlich and Langmuir models to determine where experiments with adsorbed OTC should be completed, noting a linear relationship and saturation of OTC. The linearized fit of the Freundlich isotherm gives values of K_f = 433 and n = 0.747; K_f is higher than what was found in previous study *(37)* which could be due to better sorption since these experiments use lower concentrations of OTC. Since n is less than 1, the isotherm is non-linear, indicating sorption to clay rather than organic matter. The linearized fit of the Langmuir isotherm gives values of Q = 1.1×10^{-2} mmol m^{-2} and b = 41.9 L $mmol^{-1}$ which fall within the range of OTC sorption to iron oxides in previous study *(18)*. K_d values calculated (1804 L kg^{-1} or 0.40 L m^{-2} with BET surface area 4.51 m^2 g^{-1}) are higher than a previous report due to the lower surface area of the South Farm soil *(18)*. However, these K_d

values are within the range observed in another study where soils of varying clay, organic matter content, and extractable iron were compared *(37)*.

To represent relationships between adsorption, cation exchange capacity (ECEC), and iron content (DCB Fe), we fitted our data to a previously developed model ($K_d^{est} = 19.56(ECEC) + 4.16(DCBFe) + 1373.06$) *(37)*; the estimated log K_d using values (described in previous section) found for our soil was 3.22. The measured log K_d for this soil was 3.26; therefore, the model fits well. Using our results of cation exchange capacity and iron content the estimated K_d is more influenced by the CEC value; it can be inferred that adsorption is primarily due to cation exchange capacity and not by iron complexes.

Experiments were completed at pH 7.0-7.2. At this pH, the zwitterion form of OTC has an overall neutral charge but includes a positive charge on the dimethylamino functional group *(18)*. Since this functional group has a positive charge it may be available for electrostatic interaction with the negative clay surface *(38)*.

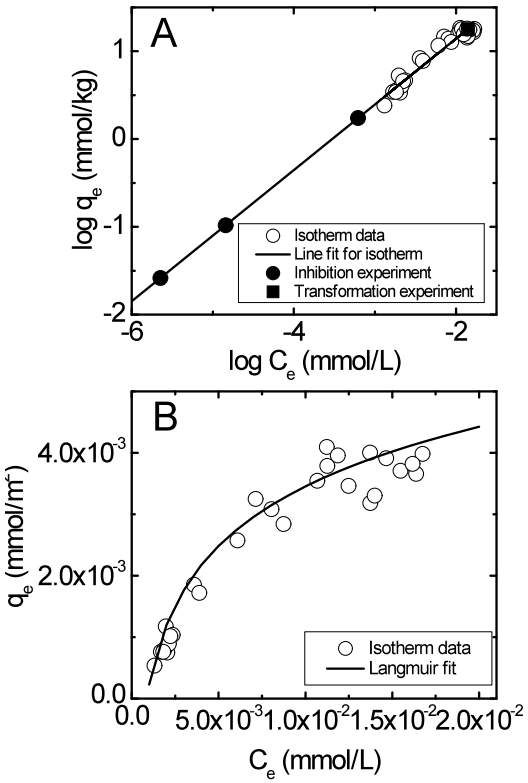

Figure 1. OTC isotherms fit to Freundlich equation (A) where $K_f = 433$ and n = 0.74 and to Langmuir equation (B) where Q (mmol/m^2) = 1.1×10^{-2}, b (L/mmol) = 41.9.

3.3 XRD ANALYSIS

Soil samples were analyzed with XRD to determine mineralogical properties and to provide understanding of the impact of OTC adsorption. The clay minerals that were identified in the South Farm soil include chlorite, kaolinite, illite, and an expandable clay; most likely mixed layer illite/smectite (I/S) (Table 2). In Figure 2, we notice the spectra change with different treatments, indicating a considerable amount of expandable clay.

Sample Name	Sample Treatment	Mineral Percentages Expandable clay	Illite	Kaolinite	Chlorite	Total Percentage
South Farm Soil-A	<2 µm gly	38%	45%	10%	8%	100%
South Farm Soil-B	<2 µm gly	32%	48%	12%	7%	100%
South Farm Soil-A	<2 µm ad	13%	70%	11%	7%	100%
South Farm Soil-B	<2 µm ad	13%	70%	11%	7%	100%
South Farm Soil-A	<2 µm htd	3%	82%	10%	5%	100%
South Farm Soil-B	<2 µm htd	5%	80%	9%	6%	100%
South Farm A-OTC	<2 µm gly	16%	66%	10%	8%	100%
South Farm B-OTC	<2 µm gly	13%	67%	12%	8%	100%
South Farm A-OTC	<2 µm ad	9%	79%	8%	4%	100%
South Farm B-OTC	<2 µm ad	7%	80%	8%	5%	100%
South Farm A-OTC	<2 µm htd	3%	85%	7%	5%	100%
South Farm B-OTC	<2 µm htd	2%	84%	9%	5%	100%

Table 2. Semi-quantitative results of clay mineral content of the South Farm samples on a 100% scale for various treatments. The changes in percentages quantify the changes in mineral peak intensities seen on the XRD diffractogram for glycolated ("gly"), air-dired ("ad"), and heated ("htd") samples.

The treatment results in Figure 2 are indicative of an I/S mixed layer clay *(39)*. EG solvation causes significant changes in the diffraction pattern as the expandable clays respond by swelling and giving a reflectance peak at the 5.2° 2θ range, which is typical of smectite being present *(39)*. Another representative peak of I/S is the reflection near the 17.7° 2θ (002/003 mineral peaks) *(39)*. After heat treatment, the expandable clays should collapse in the 5.2 - 6.1° 2θ range, similar to a pure illite diffraction pattern *(39)*. The air dried sample also shows that smectite is likely present, as the expandable peak shifts to the 6.1° 2θ range *(39)*. After OTC adsorption, a difference is seen in how the expandable clays behave after exposure to the EG. When OTC is adsorbed, it seems to inhibit the reaction of the expandable clays to EG solvation, which causes the ratio of illite to the expandable clays to change. There is a less clearly defined,

broad peak at the 5.2° 2θ range, but it does not shift to the 6.1° 2θ range. There is also a dramatic shift in the ratio of expandable clay to illite (Table 2). This indicates that the expandable clay is still able to absorb some moisture, still acting like a smectite. The presence of OTC seems to prevent the clays from swelling in the same manner as the non-OTC treated sample from EG solvation, impacting the interaction between the mixed layer clays and moisture. Similar results are seen in Table 2 in the ratio of expandable clay to illite comparison between the air dried South farm soil and the OTC air dried South farm soil. Previous study showed the presence of OTC in the interlayer space of pure smectite, an expandable clay mineral *(38)*. In our case, it was not possible to determine the distance between two clay layers due to the heterogeneous nature of the soil samples. Nevertheless, the XRD evidence that sorbed OTC significantly influenced solvation of the expandable clay suggests possible presence of OTC in the clay interlayer space.

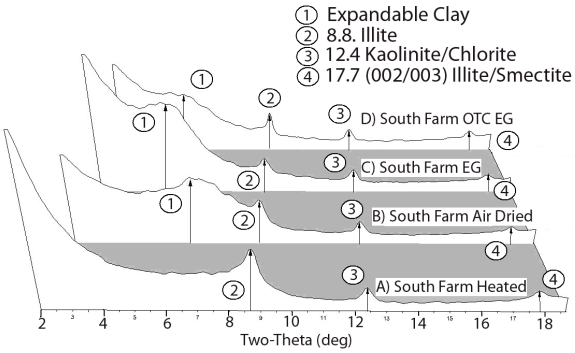

Figure 2. XRD 3-D spectra produced by different sample treatments

3.4 FTIR ANALYSIS

To further investigate the mechanism of OTC adsorption, FTIR spectra for OTC alone and the OTC-soil complex were recorded and are shown in Figure 3. Characteristic bands of OTC at the following energies were monitored: the –N-H bending bands of amide moiety at 1618 cm^{-1}, the aromatic C=C stretching band at 1583 cm^{-1}, the asymmetric CH_3 deformation band at 1456 cm^{-1}, and the C-H stretching band at 1240 cm^{-1}.

The –N-H bending band disappears after adsorption indicating that this region of the OTC molecule interacts with the soil. The aromatic C=C stretching band also disappears after adsorption. These stretching bands are associated with two phenolic –OH groups. Under our experimental conditions, these –OH groups are deprotonated, creating a potential for electrostatic interaction, cation complexation, or bridging. The asymmetric CH_3 deformation band's relative height decreased after adsorption, which may indicate an interaction between the dimethylamino groups and the soil. The appearance of a doublet of CH_3 vibration bands at 1506 and 1475 cm^{-1} following adsorption also supports an interaction with the soil. The C-H stretching band at 1240 cm^{-1} disappears after adsorption, most likely due to electrostatic interactions between the positively charged moiety on the OTC molecule and negative charges on the surface of the soil. Combining these observations and considering the experimental pH of 7.0-7.2, adsorption is most likely caused by electrostatic interactions between the protonated dimethylamino group of oxytetracycline and negative charges on the surface of the soil.

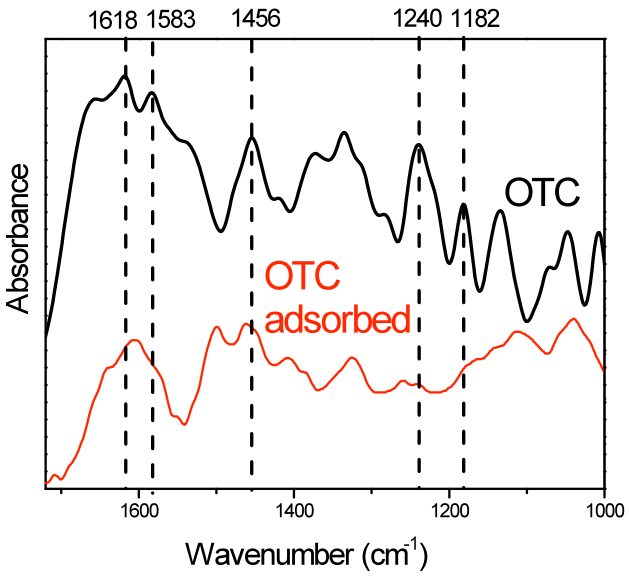

Figure 3. FTIR spectra oxytetracycline alone (black) and oxytetracycline adsorbed to soil (red).

3.5 SOIL PREPARATION AND OTC LOADING FOR ASSESSMENT OF BIOLOGICAL EFFECTS

Soil was sterilized with microwaving, autoclaving, and oven-drying to avoid biological interference. Controls validated this method, showing no growth of native bacteria from sterilized South Farm soil on B media.

Natural transformation assays include the use of DNA. Previous studies have shown that the presence of cations allows easier DNA binding to soil particles *(26, 40-42)*. Since the soil wash had very little calcium present (1.72 ppm), complexation of OTC to metals was tested by adsorbing OTC to soil in the presence and absence of $CaCl_2$. No significant difference was observed between q_e values ($p > 0.05$); therefore subsequent experiments were done with $CaCl_2$ to facilitate binding of DNA to soil.

For both natural transformation and inhibition assays, OTC was adsorbed to soil. Initial amounts of OTC were 250 µg and 1250 µg. $q_{e,2}/q_e$ values for initial amounts near 250 µg ranged

from 78-93%, with an average of 87% and standard deviation of 4%. For initial amounts near 1250 μg, $q_{e,2}/q_e$ values ranged between 50-75%, averaging 58% with a standard deviation of 8%. These loadings agree with previous study that found soil loadings of OTC were on average 85% *(37)*.

3.6 NATURAL TRANSFORMATION OF *A. VINELANDII*

3.6.1 *Without OTC*

Because the natural transformation assay was developed in sterile conditions, we first needed to adapt it for use with soil. Transformation experiments without any OTC present were done to determine if adsorbed, compared to dissolved, plasmid DNA will transform *A. vinelandii*. Average frequencies for conditions without soil ($2.4\times10^{-6}\pm1.4\times10^{-6}$) and with soil ($4.5\times10^{-6}\pm2.3\times10^{-6}$) confirm that adsorbed plasmid DNA does transform, and that the transformation frequency for adsorbed plasmid DNA is not significantly different from dissolved DNA ($p > 0.05$).

Homology between the plasmid and chromosomal DNA was also investigated for its effect on transformation frequency *(43)*. The plasmid pDB17 includes the *nifH* gene fragment 208-1984 from the *A. vinelinadii* DJ chromosome *(32)*. Average frequencies for dissolved experiments with pDB17 ($2.4\times10^{-6}\pm1.4\times10^{-6}$) and with pBR325 ($1.4\times10^{-6}\pm7.2\times10^{-7}$) show no significant difference ($p > 0.05$); therefore, homology did not affect transformation frequency. Since transformation work was more reliable with plasmid pDB17, the transformation assays comparing OTC concentrations were completed with pDB17.

3.6.2 *Dissolved OTC*

Because tetracyclines can increase the probability of transferring genetic material by some transposons *(44, 45)*, the effect of OTC on natural transformation frequency was investigated. Transformation experiments without soil were done with OTC concentrations

ranging from 0 μg/ml to 70 μg/ml, which includes the minimum inhibitory concentration (16 μg/mL) *(46)* and environmentally relevant concentrations. Transformations occurred throughout this range of concentrations (Figure 4), with the frequency of $3.8\times10^{-6}\pm2.4\times10^{-6}$ observed at the highest concentrations. This work shows that the amount of OTC dissolved did not significantly influence transformation frequency. The molecular mechanism responsible for allowing gene transfer in the presence of tetracycline was specific to this type of transposon *(44, 45)*. Attenuation does not occur in natural transformation; therefore it makes sense that tetracyclines did not influence gene transfer from natural transformation. OTC does not affect natural transformation.

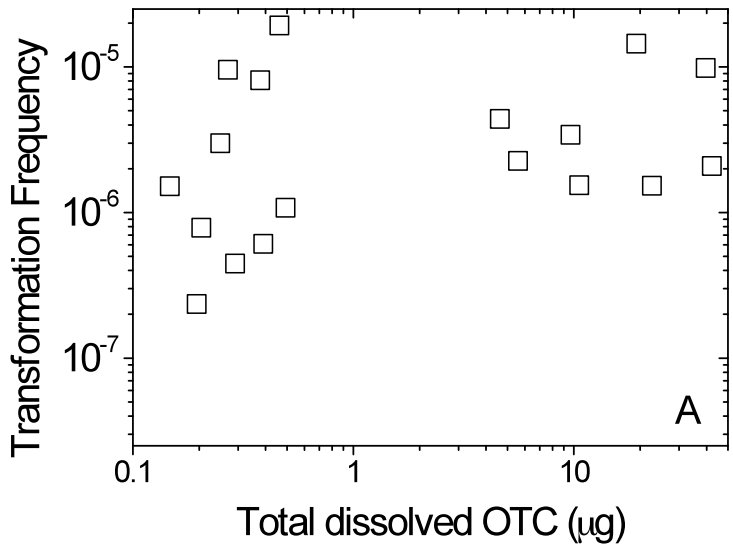

Figure 4. Natural transformation of *A. vinelandii* with dissolved OTC. Transformation experiments were conducted with plasmid DNA pDB17 at various concentrations of dissolved OTC. Data are presented to compare the total amount of OTC available (A) and the dissolved OTC concentration (B).

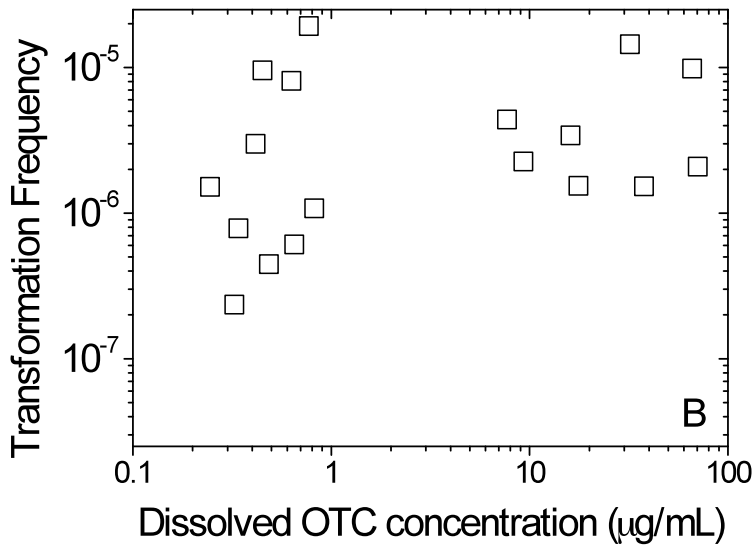

Figure 4 (cont.). Natural transformation of *A. vinelandii* with dissolved OTC.

3.6.3 *Adsorbed OTC*

When compared to dissolved conditions, adsorbed OTC and DNA did not significantly affect transformation frequency. The results of transformation experiments with varying OTC amounts adsorbed to soil are shown in Figure 5. OTC was adsorbed at concentrations ranging from 0 μg/g of soil to 8200 μg/g of soil, which includes environmentally relevant concentrations. Transformations were observed throughout this range, with the frequency of $3.5\times10^{-6}\pm5.0\times10^{-7}$ at the highest concentration. The amount of OTC present in the experiment did not influence transformation frequency.

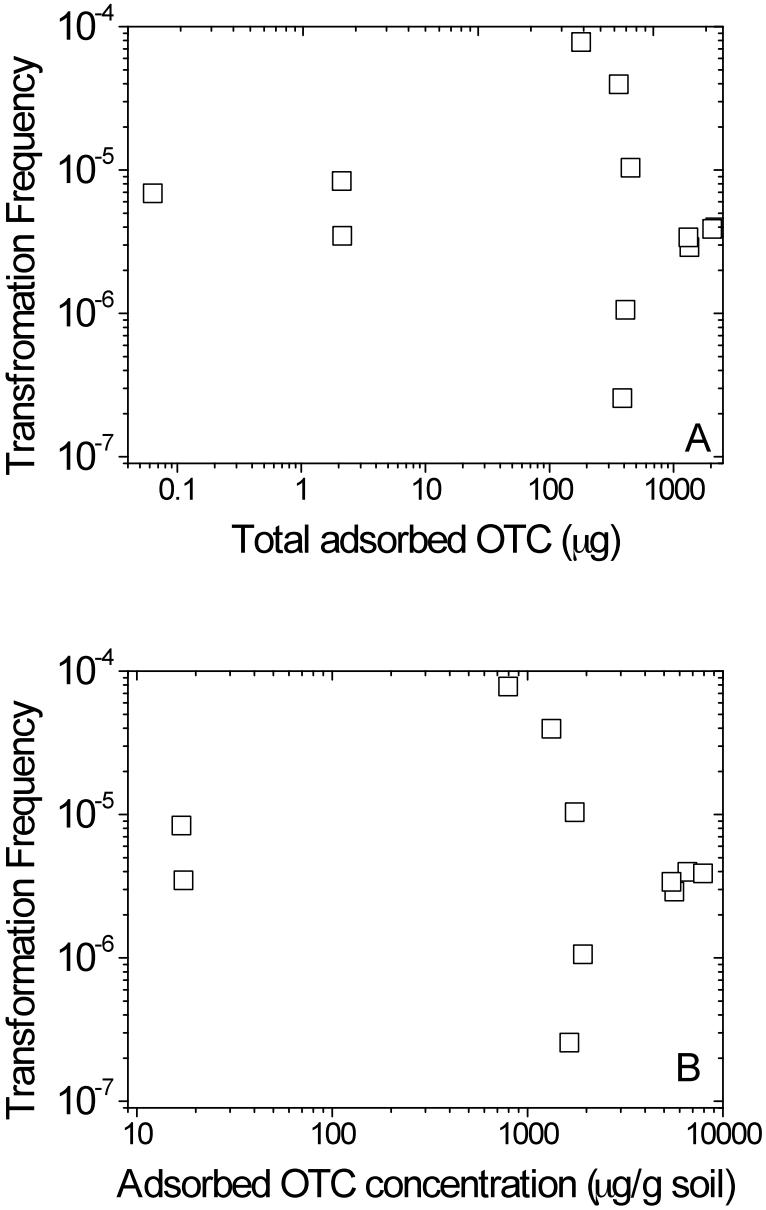

Figure 5. Natural transformation of *A. vinelandii* with adsorbed OTC. Transformation experiments were conducted with plasmid DNA pDB17 at various concentrations of OTC adsorbed to soil. Data are presented to compare the total amount of OTC available (A) and the adsorbed OTC concentration (B).

3.6.4 *Chromosomal DNA*

Natural transformation assays with chromosomal DNA were completed with and without OTC dissolved and adsorbed. Transformation frequencies without OTC were found: $3.5\times10^{-}$

$^{3}\pm 7.8\times10^{-5}$ (dissolved) and $1.2\times10^{-3}\pm 8.1\times10^{-4}$ (adsorbed). Frequencies for experiments with OTC are shown in Figure 6. Frequencies were not influenced by the addition of OTC, either dissolved or adsorbed to soil. This is consistent with our findings for plasmid DNA.

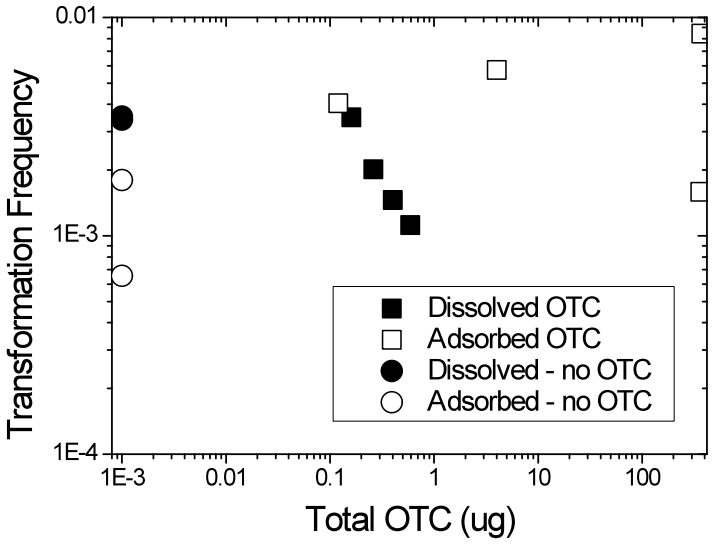

Figure 6. Natural transformation of *A. vinelandii* with chromosomal DNA and OTC present. Experiments were conducted with chromosomal DNA at various OTC concentrations. Each data point depicts one experiment. Data are presented individually to compare experiments with and without OTC and with and without soil present.

3.7 CHROMOSOMAL DNA TRANSFORMATION WITH RIF^{R} DNA

Natural transformation assays without OTC were completed with rifampin resistant chromosomal DNA selecting for rifampin resistance. Frequencies of $4.75\text{x}10^{-5}$ and $3.61\text{x}10^{-5}$ were found for a single experiment.

3.8 GROWTH INHIBITION OF *AZOTOBACTER VINELANDII*

Inhibition experiments were conducted with two different total OTC amounts. 6 μg of OTC was the lowest amount that inhibited growth in growth curves. 100 μg of OTC corresponds to the minimum inhibitory concentration (MIC) for resistances of bacteria family to *A. vinelandii* (MIC for *Pseudomonas aeruginosa* is 16 μg/ml *(46)*; total volume of inhibition assay was 6.55 ml). Results for inhibition experiments are shown in Figure 7. As confirmed in another study *(3)*, dissolved OTC inhibits growth significantly more than adsorbed OTC. OTC enters the cell by chemical force diffusion across the cell membrane as a cation-OTC complex through the Donnan potential *(47)*. From our FTIR results, we can see that the dimethylamino functional group (that has a positive charge) interacts with the negative clay surface for adsorption *(38)*. From our XRD results, we see that OTC adsorbs inside the expandable clay layers and is therefore less available to inhibit the growth of bacteria, which is evident in our inhibition results. However, inhibition is still occurring even when OTC is adsorbed at higher values.

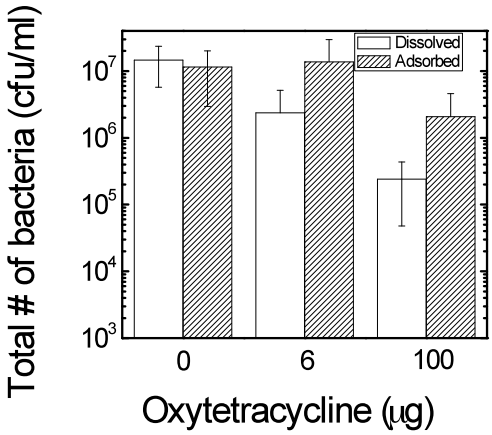

Figure 7. Inhibition experiment at varying OTC amounts. Inhibition experiments were done at important OTC concentrations: 6 μg corresponds to minimal growth in growth curves, and 100 μg corresponds to the minimum inhibitory concentration for resistance of tetracyclines for bacteria family to *A. vinelandii*.

CHAPTER 4

CONCLUSIONS

OTC is a strongly adsorbed antimicrobial that is present in the environment due to manure application to farm fields *(1, 9-11)*. The soil used in this work is considered the state soil, being the most productive soil in the state for agriculture and covering more than 1.5 million acres of the state *(25)*. OTC adsorbs inside the expandable clay layers of the soil. Our results that OTC adsorbed to this soil is still effective at inhibiting the growth of soil bacteria suggest possible contribution to the selection of antibiotic resistant bacteria. In addition, the presence of OTC in either dissolved or adsorbed form was found not to hinder gene transfer. Thus, adsorbed OTC can contribute to a change in microbial communities as reflected by previous study that showed increased antibiotic resistance genes in newer soils *(20)*.

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APPENDIX A

NATURAL TRANSFORMATION OF NON-STERILE SOIL USING GFP

A.1 INTRODUCTION

The transfer of genetic material can occur through natural transformation of bacteria. With the addition of genes to the soil environment through land application of manure, natural transformation is a possible mode of transferring genetic material which may contribute to antibiotic resistance in the natural soil environment.

Much research has explored gene transfer into cultured soil bacteria. In one study, microcosms of non-sterile soil were used to investigate natural transformation of *Pseudomonas stutzeri* by DNA with a *his*$^+$ marker *(48)*. The soil was loaded first with DNA, followed by competent cells. This study showed that DNA bound to non-sterile soil can transform and survive native DNases. In another study, chromosomal and plasmid DNA bound to the clay mineral montmorillonite were again able to transform bacteria *(49)*. These DNA-clay complexes were found to transform the bacterium *Bacillus subtilus* even 15 days after being introduced to a non-sterile soil environment. This work supports that DNA bound to clay minerals is able to resist degradation and is therefore available for transformation.

In order to study the uptake of genetic material, the transfer must be monitored. The green fluorescent protein (GFP) has been established as a good tracker for microorganisms. Horizontal gene transfer was monitored using a plasmid encoding GFP to investigate natural transformation of the bacteria *Acinetobacter* sp. strain BD413 in a biofilm *(50)*. Transformations occurred most frequently with young and active biofilm growth. In addition, only 15 minutes of exposure to DNA was needed for successful uptake of DNA. In another study that aimed to

monitor fungal growth and activity in native soils, GFP was used as a non-destructive method of determining transformant hyphae in native soil *(51)*.

The versatile use of *gfp* provides the potential to track natural transformation in the soil environment. This study's aim was to investigate natural transformation of native soil bacteria using the plasmid pAKgfp1, which encodes GFP *(52)*. The detection of GFP is explored through microscope and microplate protocols. The transformation frequency of the *gfp* plasmid into a soil bacterium, *Azotobacter vinelandii,* was measured to determine an expected frequency for native soil bacteria. Since the frequency averages $3.6x10^{-5}$ for *A. vinelandii*, and the microscope detection limit is 200-300 cells per view, my conclusion is that using *gfp* as a tracker for natural transformation in this system is infeasible.

A.2 MATERIALS AND METHODS

A.2.1 BACTERIAL STRAINS, CULTURE MEDIA AND GROWTH

The *E. coli* strain S17 with plasmid pAKgfp1 was used as the positive control for fluorescent detection. The same *E. coli* strain without the plasmid was used as the negative control. The *gfp* strain was cultured in LB media with carbenicillin (50 μg mL^{-1}). The dark strain was cultured in LB media alone. Liquid cultures were grown at 37°C for 16-18 hours while shaking at 250 rpm. To induce fluorescence, isopropyl β-D-1-thiogalactopyranoside (IPTG, 100 mM) was added after 16 hours of growth and continued incubation at 37°C for two hours.

As another control, the plasmid pAKgfp1 was transformed into the cultured soil bacterium *Azotobacter vinelandii*. Wild type *A. vinelandii* strain DJ was used as a recipient (source Dr. Dennis Dean). The strain was cultured in Burk's media at 30°C with 170-rpm shaking *(53)*. Burk medium with 0.013 mol L^{-1} ammonium acetate (BN medium) was also used.

A.2.2 DNA PURIFICATION

Plasmid DNA pAKgfp1 was purified from the *E. coli* strain S17. The culture was grown at 37°C for 16-18 hours, and the plasmid was prepared following the QIAGEN Plasmid Maxi Kit protocol. Plasmid DNA was suspended in endotoxin-free water and stored at -20°C. The concentration was determined with UV spectroscopy and quantified with agarose gel electrophoresis.

A.2.3 NATURAL TRANSFORMATION ASSAYS

For competence wild type *A. vinelandii* DJ was grown on BN plates without molybdenum at 30°C for two days, inoculated into BN liquid media and grown at 30°C for 18-20 hours with 170-rpm shaking. 200 μL of competent *A. vinelandii* cells, 200 μL of MOPS transformation buffer (20mM morpholinepropanesulfonic acid with 1mM $CaCl_2$ transformation buffer dissolved in nanopure water and adjusted to pH 7.0-7.2 with NaOH) and 10 μg of DNA were incubated at room temperature for 20 minutes. 100 μL of the dissolved transformation mixture was added to 2.4 mL of B media and mixed for 17 hours at 30°C. This solution was diluted with phosphate buffer (0.16 M KH_2PO_4 and 0.51 M K_2HPO_4 dissolved in DI water) and 50μL was spread on both B media with carbenicillin at 20 μg mL^{-1} and B media. The plates were incubated at 30°C for 4 days. Transformation frequencies were determined by dividing the number of transformants (colonies on B with carbenicillin plates) by the number of viable cells (colonies on B plates).

A.2.4 MICROSCOPE SLIDE PREPARATION – PURE CULTURE

A six-well slide was loaded with 5 μL of suspended bacterial solution. The slide was air dried while covered to protect from light. After the slide was dry, DAPI (4',6'-diamidino-2-phenylindole, 2 μg mL^{-1}) stain was added to cover the area of the slide (<300 μL). The slide was

covered and stained for 5-10 minutes. The slide was rinsed with 1-5 mL of DI water, covered, and air dried again. Once the slide was dry, a drop of Citifluor was added, and a cover glass was secured on top with clear nailpolish outlined on the sides. Citifluor was used to allow 100x magnification and to preserve the fluorescent properties of the sample as an anti-fading agent.

A.2.5 MICROSCOPE SLIDE PREPARATION – FILTER

Using a filter apparatus, the bacterial solution was filtered through a 0.2 μm polycarbonate filter. While covering the apparatus, 1-2 mL of DAPI stain was added to make sure the entire filter was exposed to the stain for 5-10 minutes. Following the staining period, 2-5 mL DI water was added, and the vacuum was used to rinse the filter. The filter was removed from the apparatus and placed on a glass slide. A drop of Citifluor was added, and a cover glass was secured on top with clear nailpolish outlined on the sides.

A.2.6 MICROPLATE READER

To investigate the time dependence of GFP expression, a Molecular Devices SpectraMax Gemini Spectrophotometer microplate reader was used. After 16 hours of culture growth, three cultures of *E. coli* were monitored. A culture without the pAKgfp1 plasmid (denoted as "dark"), a culture with the pAKgfp1 plasmid ("gfp"), and a culture with the pAKgfp plasmid and IPTG added ("IPTG") were observed by the microplate reader. 100 μL of culture were added to one of the wells in a 96 well black polystyrene plate. 5 replicate cultures were monitored for each assay. Detection was at an excitation of 458 nm and emission at 538 nm.

A.3 RESULTS

A.3.1 MICROSCOPE – PURE CULTURE

Figures A1 and A2 show results of spotting bacterial solution on a slide and viewing the slide under the DAPI and FITC filter sets. Bacteria expressing *gfp* are seen on the FITC image.

Figure A1 shows images of *E. coli* without the plasmid. We do not expect to see the bacteria using the FITC filter. Figure A2 shows images of *E. coli* with the pAKgfp1 plasmid. We expect to see the bacteria on both the DAPI and FITC image.

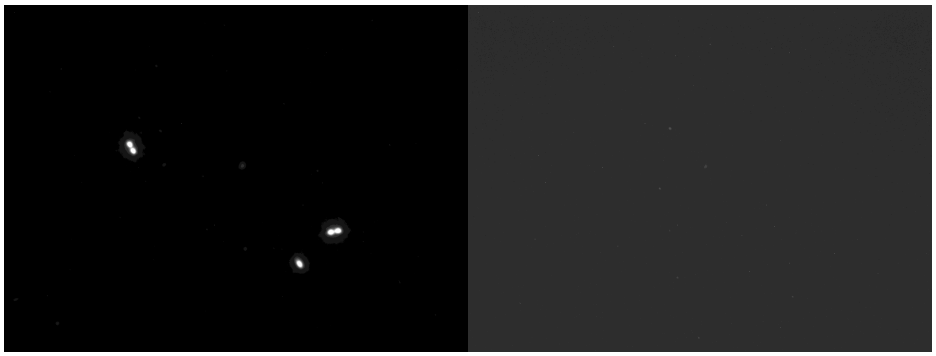

Figure A1. DAPI image of pure culture of *E. coli* -dark (left). FITC image of the same field for control, showing no *gfp* expressing bacteria (right).

Figure A2. DAPI image of pure culture of *E. coli* pAKgfp1 (left). FITC image of the same field showing *gfp* expressing bacteria (right).

A.3.2 MICROSCOPE – FILTRATE OF PURE CULTURES

Figure A3 shows images of viewing the 0.2 μm filter paper after *E. coli* with pAKgfp1 has been collected on the filter.

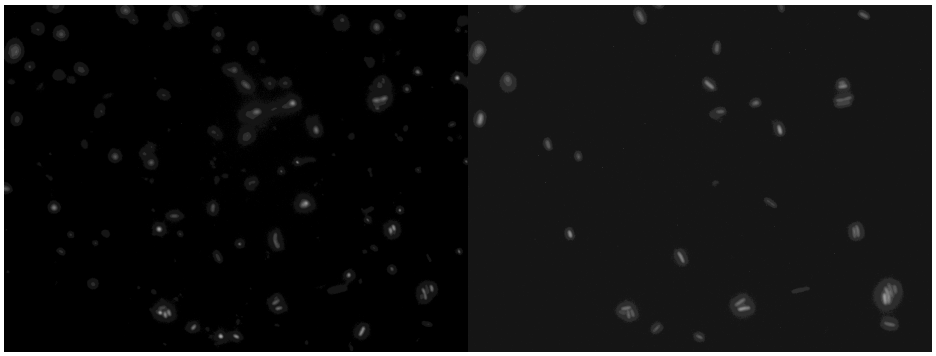

Figure A3. DAPI image of pure culture of *E. coli* pAKgfp1 on a filter (left). FITC image of the same field showing *gfp* expressing bacteria (right).

The bacteria are more concentrated using the filter paper for viewing bacteria under the microscope (i.e. ~40 cells are seen). The filtrate method of preparing slides will be used to detect *gfp*-expressing bacteria when mixed with soil wash solution.

A.3.3 MICROPLATE READER

Figure A4 shows the results of fluorescence as quantified by the microplate reader. The results validate the use of IPTG to enhance the *gfp* fluorescence viewing. The IPTG assay shows greater intensity with time than the dark control and the *gfp* without IPTG added.

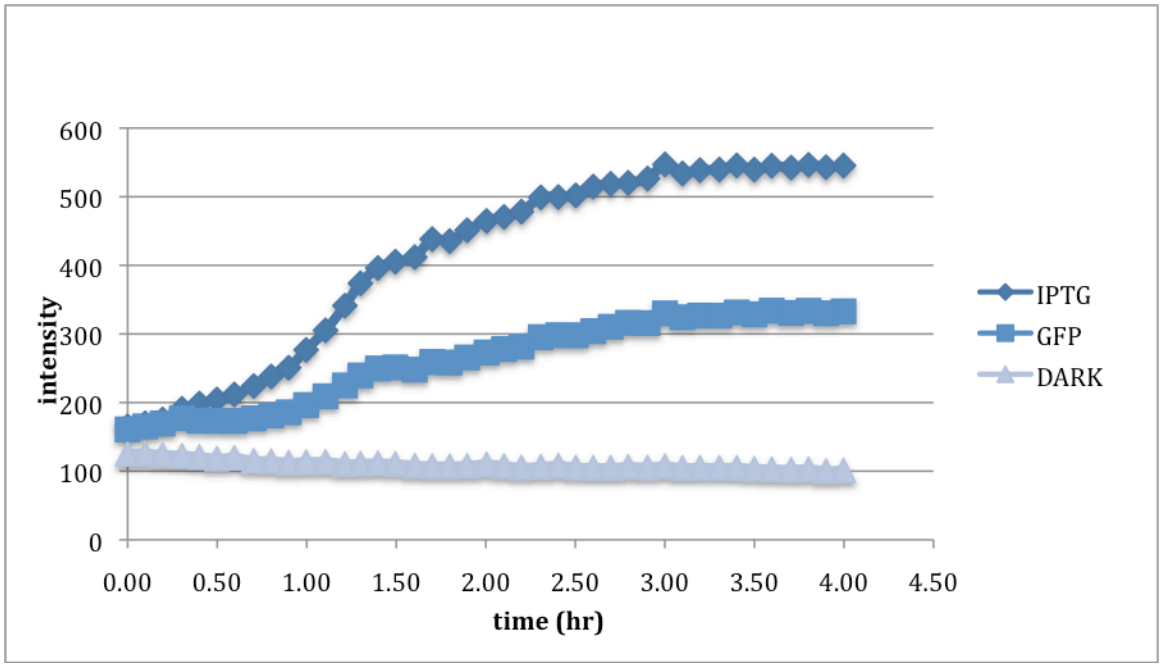

Figure A4. Fluorescent intensity of *E. coli* without plasmid pAKgfp1 (dark), *E. coli* with plasmid pAKgfp1 (gfp), and *E. coli* with plasmid pAKgfp1 with IPTG added (IPTG).

A.3.4 NATURAL TRANSFORMATION OF A. VINELANDII BY PAKGFP1

Natural transformation of *A. vinelandii* was completed to determine if plasmid pAKgfp1 would transform a cultured soil bacterium. The average transformation frequency of duplicate experiments was 3.6×10^{-5}.

A.4 DISCUSSION AND CONCLUSION

Even though the microplate reader confirmed the *gfp* plasmid's successful fluorescence, the objectives of this work are dependent on a successful microscope detection method. Since soil will be present in these assays, the microplate reader would not be able to distinguish background fluorescence of the soil from *gfp* fluorescence. Preliminary transformation data with a cultured soil bacterium *Azotobacter vinelandii* with the pAKgfp1 plasmid resulted in a transformation frequency of 3.6×10^{-5}. For the microscope detection method to observe transformants, more than a million cells would need to be viewed. We would expect

transformation of the cultured *A. vinelandii* to be a more "ideal" situation as compared to using uncultured, native soil bacteria. Native soil bacteria may transform the pAKgfp1 plasmid, but perhaps not as frequently, resulting in infeasible viewing of more than a million cells for each replicate of an experiment. Following this conclusion, the scope of this work was modified.

APPENDIX B

ADSORBED DNA PREPARATION

B.1 INTRODUCTION

Naked or extracellular DNA is known to adsorb to soil particles in a natural setting. To mimic the natural soil environment, adsorbed DNA was used in soil batch experiments. It is necessary to quantify the amount of adsorbed DNA; this is explored through detecting the amount of DNA not adsorbed (dissolved in solution) and applying mass balances.

To detect DNA in solution, two methods were considered: the Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and the Qubit fluorometer (Invitrogen, Carlsbad, CA). The purpose of this appendix is to determine the best way to detect DNA in the presence of soil.

The Nanodrop is a highly sensitive UV spectrophotometer and excels at detecting pure DNA in solution from low sample volumes. The Qubit uses fluorescent reagents that only bind to DNA. The Qubit may have a lower detection limit than the Nanodrop in the presence of natural organic matter, since it can remove interference from soil.

B.2 MATERIALS AND METHODS

B.2.1 CHEMICALS AND REAGENTS

The transformation buffer, 1XMOPS, was used and consisted of 1 mM $CaCl_2$ and 20 mM MOPS (pH=7.2). This buffer was used to dilute DNA for calibration curves and to suspend the soil and DNA mixture. Salmon sperm DNA (Fisher Scientific) and Herring sperm DNA (Promega) were used as adsorbates. Soil was a Drummer silty clay loam sampled from the South Farms of the University of Illinois; a detailed characterization is presented in the thesis.

To sterilize the soil, a 5 g aliquot of soil was microwaved for 5 minutes and autoclaved for 60 minutes.

B.2.2 NANODROP UV SPECTROPHOTOMETER

The Nanodrop UV spectrophotometer was used to detect DNA. After loading 2 μL of sample onto the device, the output reading was an A260 value. These values were converted to a concentration using a calibration curve.

B.2.3 QUBIT FLUOROMETER

The Qubit fluorometer was used to detect DNA by preparing assays. 0.5 mL PCR tubes were needed for two standards and for each sample. The working solution was prepared by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. 200 μL of working solution were required for each sample and standard. Once the working solution was created, 190 μL of working solution were added to each PCR tube. To each tube, 10 μL of standard or sample were added. All tubes were then vortexed for 2-3 seconds and then incubated at room temperature for 2 minutes. The tubes were then placed in the fluorometer to be read. Following calibration of the instrument with the standards, the rest of the samples were read. The output reading is a "QF value" which was then converted to the concentration of DNA in sample using the equation:

Concentration of sample = QF value * (200/X)

Where

QF = value given by Qubit fluorometer

X = volume (μL) of sample added to assay tube

B.2.4 DNA ADSORPTION

Salmon and herring sperm DNA were used for adsorption experiments. Transformation buffer was used to dilute DNA. 0.25 g of soil was used as the medium for DNA to adsorb.

Three controls were used, and their purposes are summarized in the Table B1. The X value corresponds to the initial concentration of DNA (C_e).

Composition	**Purpose**
1 mL MOPS buffer + 0.25 g soil	Background interference
1 mL MOPS buffer + 0.25 g soil + X μg/ml DNA	Amount of DNA not adsorbed to soil (in solution)
0.5 mL MOPS buffer + X/2 μg/ml DNA	Initial amount of DNA in solution

Table B1. Controls.

Five replicates of controls were completed. Buffer, soil, and DNA (where applicable) were rotated for 24 hours in 2 mL tubes. After rotation, tubes were centrifuged at the highest setting (13,200 rpm) for 1 minute. The supernatant was decanted into fresh tubes and sampled on the Nanodrop. MOPS buffer was used to blank the spectrophotometer. The interference value from the control was subtracted from the amount of DNA not adsorbed. These values once calibrated were used to calculate the amount of DNA adsorbed. Four different initial values (C_e) were used: 25, 50, 100, and 200 μg/ml DNA.

B.2.5 DNA DESORPTION

DNA may desorb from the soil assay. To test for this, after the DNA solution were decanted, the remaining soil and DNA adsorbed to the soil was immersed in 1 mL of 1XMOPS buffer, shaken until resuspended again, and centrifuged at 13,200 rpm for 1 minute. The supernatant was decanted to a fresh tube. This solution was analyzed by the Nanodrop and Qubit to detect any desorbed DNA. If the readings show DNA in solution, the process is repeated again to minimize the amount of desorbed DNA.

B.3 RESULTS AND DISCUSSION

B.3.1 CONSISTENCY OF SOIL AND BUFFER READING

Since soil is being used as the medium for DNA adsorption, it was important to check whether it would result in inconsistent readings. This was tested by rotating different volumes of buffer and masses of soil overnight and centrifuging the resulting solution. From these results, the supernatant from the 1 mL MOPS buffer with 0.25 g sample gave the most consistent results, taking into account that low amounts of soil and buffer were desired.

B.3.2 DNA ADSORPTION

The Nanodrop UV spectrophotometer was used to determine the amount of DNA in solution following adsorption experiments. Calibration curves with known concentrations of DNA were completed. Using mass balance analysis, the amount of DNA adsorbed to soil can be determined. Figure B1 shows the results of comparing different experiments at similar conditions using salmon and herring DNA.

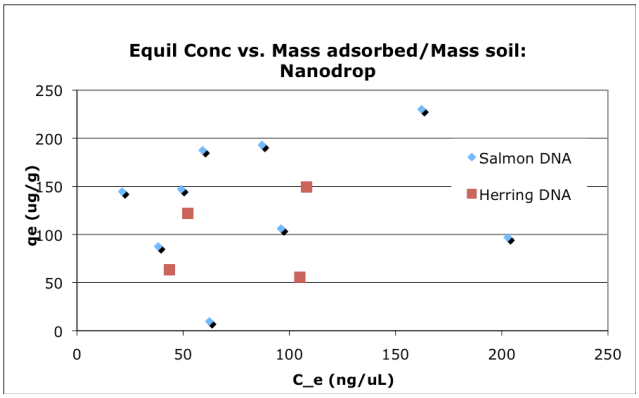

Figure B1. Isotherm data for salmon and herring DNA using Nanodrop (from file adsorptionsummary.xls).

Inconsistent values resulted when using salmon DNA; I believe the variability was either due to freezing and thawing degrading the salmon DNA or that soil interference may have caused inconsistent readings. Therefore, new DNA was purchased. Herring sperm DNA from

Promega was used and aliquoted in smaller volumes to minimize degradation due to freeze/thaw cycles. The Qubit fluorometer was also used after these results to minimize possible interference from the soil.

B.3.3 CALIBRATION CURVE: QUBIT

A calibration curve for the fluorometer was also developed. The inconsistent values that resulted are shown in Figure B2. This data may indicate that the Qubit is not useful for detecting low concentrations of DNA, but possibly sufficient at higher concentrations.

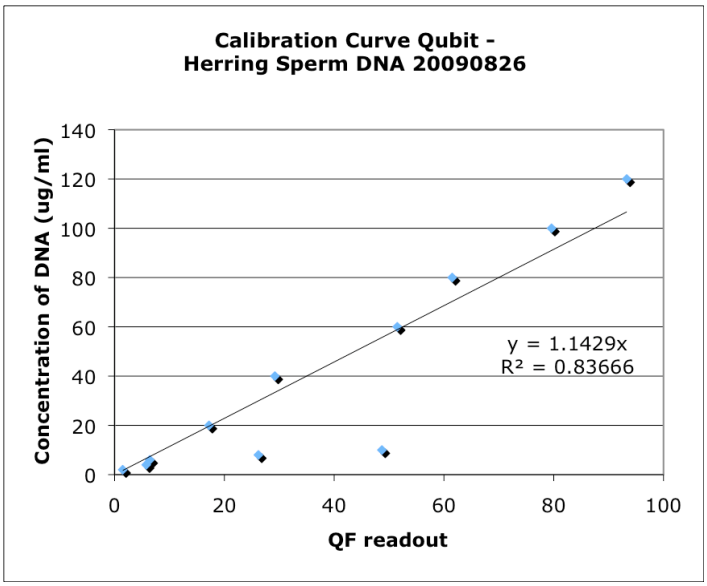

Figure B2. Calibration curve of Herring Sperm DNA using Qubit (from file 20090826calcurveherringdna.xls).

B.3.4 DNA DESORPTION

The supernatant after one wash indicates desorbed DNA. Using both the Nanodrop and Qubit, we can compare the readings to determine the best instrument to use. Table B2 shows a difference in detection between the UV spectrometer and the fluorometer. The Nanodrop readings give unexpected results, showing similarity of values between the different conditions;

this may be the background signal for soil interference. This data also shows that all DNA has desorbed, which isn't expected. The Qubit data suggests that soil is not interfering, as we are seeing more DNA in the wash after 100 μg/ml of DNA has been introduced to the system, which is an expected result. A background level of interference is seen from the soil reading, however it is less than the experiments with DNA, suggesting that the Qubit is better at detecting desorbed DNA.

Concentrations after one wash (from file 20090901adsexpherringdna50and100.xls)

	50 μg/mL	100 μg/mL	soil
Qubit	2.68±0.48	7.54±0.39	1.69±0.17
Nanodrop	49.55±6.09	55.65±9.50	53.66±4.19

Table B2. Desorption data converted to concentration (μg/mL) comparing values between Qubit and Nanodrop.

B.4 CONCLUSIONS

The Nanodrop has a high detection limit, but sufficient DNA will be used in our experiments; therefore using the Nanodrop for detection of adsorbed DNA is appropriate. The Qubit performs better where the Nanodrop lacks. According to the wash data, the Nanodrop reports no DNA adsorbed, i.e. all DNA has been washed. However the Qubit data confirms that there is still DNA in solution. The Qubit data is also more consistent (i.e. lower standard deviation) than the Nanodrop in the desorption data.

From this analysis, the Nanodrop spectrophotometer will be used for detecting DNA in solution, to determine the amount of DNA that has adsorbed to soil. The Qubit spectrofluorometer will determine if there is any desorption of DNA and whether or not more wash sequences need to be conducted.