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Assessment of Key Parameters to Achieve Bacteria Transformability in *E. coli* and *A. tumefaciens*

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Abstract

In this project, growth and transformability of *E. coli* and *A. tumefaciens* were investigated. The growth of the bacterial species was monitored under different key parameters including: the strain of the bacteria, the inoculum source (a fresh plate or frozen glycerol stock), and the media type. Growth was monitored by measuring the optical density (OD). Using *E. coli* strains Dh5α and CdB^R, it was determined that the strain, the media type, and the amount of inoculum had a great effect on the growth but the source of the inoculum had little effect. For *A. tumefaciens*, the strain and the media type had a great effect on the growth and the source of the inoculum had a great effect for one strain's growth but little effect for the other.

Transformation is a process by which cells take up DNA from their environment. For this project, the artificial transformation process of electroporation was utilized in order to transform cells by exposing a mixture of cells and free DNA to a strong electrical field. Bacteria grown to different phases of growth under various conditions were used for electroporation and transformation efficiencies were determined. Trends that were observed included higher transformation efficiencies for the $CcdB^R$ stain than the Dh5α strain of *E. coli*, higher transformation efficiencies for 0.4 OD cultures than for 0.2 OD cultures, and no significant effect of the media type on transformation efficiencies. Standard deviations were too large for these results to be conclusive.

Introduction

Electroporation is a genetic transformation process in which a strong electric field is used to introduce DNA into cells. (Tortora, 2004) Bacterial transformation is important for molecular cloning, gene construct selection, and plasmid production and storage (Lewin, 2004). Bacterial transformation is also important in plant transformation. (Snyder, 2003). Two species of bacteria of particular interest are *Escherichia coli* and *Agrobacterium tumefaciens*. As the "workhorse" of molecular biology, *E. coli* is important in molecular cloning, gene construct selection, and plasmid production and storage. *A. tumefaciens* is important in plant molecular biology for its natural ability to transform DNA into certain plants, many of which are more successfully transformed by this than method than by gene gun transformation procedures(Snyder, 2003). The strains that were selected for this study were the "regular", well-known stains and the strains of more particular interest for other research in Iowa State's Plant Transformation Facility. Dh5α and LBA4404 were the well-known strains for *E. coli* and *A. tumefaciens* respectively (Dh5α, 2006). $CcdB^R$ and EHA were the strains of particular interest to the Plant Transformation Facility. $CcdB^R$ has applications in Gateway cloning due to its resistance to the CcdB toxin used and EHA has applications for its increased cloning stability (One shot ccdB, 2004).

To optimize the electroporation process, several key parameters were investigated, including: the strain of the bacteria, the inoculum source, and the media type. Bacteria were grown under different conditions and their growth was monitored. Bacteria grown to different phases of growth under various conditions were used for electroporation and transformation efficiencies were determined.

Since information about the growth of the bacteria was important to the electroporation procedure, optical densities (ODs) of the growing bacteria cultures under the different parameters was monitored in short time intervals over the course of their growth, ideally until the growth markedly leveled off. This information was graphed using Microsoft Excel and this growth curve information was used to determine differences in growth for cultures grown under different parameters. It was also useful in determining the time needed to grow cultures to the particular ODs they would be used at for the transformation procedure. It also allowed for those ODs to be matched up with the cultures phase of growth on the growth curve, an indication of the health of the bacteria (with earlier stages in the growth curve being healthier and having more nutrients available and later stages being less healthy with more waste present).

Methods

Monitoring the Growth Curve:

The growth curves of the *E. coli* strains DH5α and CcdB^R and the *A. tumefaciens* strains LBA4404 and EHA101 were determined by measuring optical densities (ODs) and plating diluted culture samples and counting colonies to determine colony forming units / mL. Growth curves were determined for cultures grown from plate and glycerol inoculums. Growth curves were also detemined for *E. coli* grown in LB and 2xTY media and *A. tumefaciens* grown in LB and YEP media. (see Figure 1)

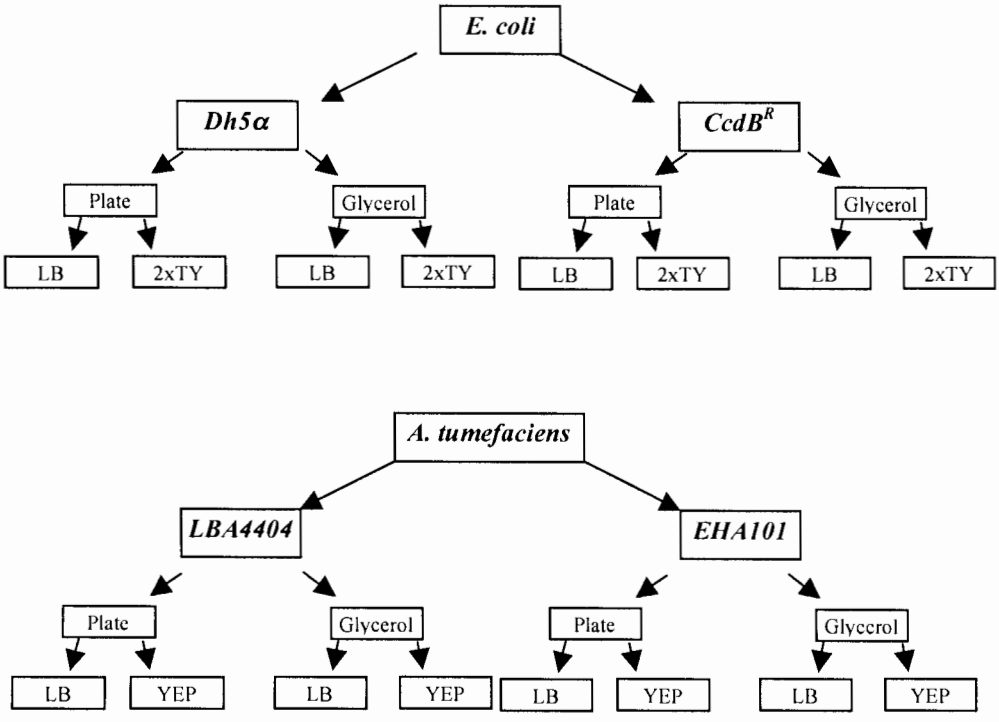

Figure 1 : Key Parameters for Bacterial Growth

The first step in this process was to start pre-cultures to inoculate the cultures. For the plate inoculum, a plate was streaked and grown overnight in an incubator (37°C for *E. coli* and 28°C for *A. tumefaciens*). A single colony was picked with a sterile

toothpick and the toothpick was put into a 15 mL culture tube containing 3 mL liquid LB medium. After about 6 hours of growth, 500 µL of this initial pre-culture was transferred into a 50 mL Falcon screw-top tube with 20 mL fresh liquid medium (of the type that the bacteria would be grown in the following day) and the tube was incubated at the appropriate temperature with the tube on its side shaking at 250 rpm. For the glycerol inoculum, a frozen glycerol culture was retrieved from the -80°C. A small amount was scraped from the top with a sterile toothpick which was put into a 15 mL culture tube containing 3 mL liquid LB medium. After about 6 hours of growth, 500 µL of this initial pre-culture was transferred into a 50 mL Falcon screw-top tube with 20 mL fresh liquid medium (of the type that the bacteria would be grown in the following day) and the tube was incubated at the appropriate temperature with the tube on its side shaking at 250 rpm.

The following morning, the optical densities of the overnight pre-culture was measured for a wavelength of 610 nm with a spectrophotometer zeroed with fresh media. 1mL of culture in a new or recycled cuvette was used to measure the OD, and 3 measurements were taken per time point. 300 mL of media (in a 1 L bottle for *E. coli* and in a 1 L flask for *A. tumefaciens*) was inoculated with 20 mL of overnight pre-culture diluted to an optical density of 0.5. After inoculating, the culture was incubated in a 250 rpm shaker at the appropriate temperature (37°C for *E. coli* and 28°C for *A. tumefaciens*). For the *E. coli* strains, the OD was measured every 15 minutes and a plate was spread from 100 µL of diluted culture every 30 minutes. Several dilutions were tried and the dilution that worked the best was a 1×10^{-4} dilution made by transferring 100 µL culture into 1 mL of LB, then transferring 100 µL of that culture into another 1 mL of LB liquid medium, vortexing between each transfer. Plates were incubated at 37°C until colonies

were counted the following day. For the *A. tumefaciens* strains, the OD was measured and a plate was spread from 50 µL of diluted culture every 40 minutes. Several dilutions were tried and the dilution that worked the best was a 1×10^{-6} dilution made by transferring 100 µL culture into 1 mL of LB, then transferring 100 µL of that culture into another 1 mL of LB, then transferring 100 µL of that culture into another 1 mL of LB liquid medium, vortexing between each transfer. Plates were incubated at 28°C until colonies were counted 2 days later.

Data was analyzed using Microsoft Excel.

Electroporation

Electroporation was carried out on *E. coli* Dh5α and $CcdB^R$ strains and *A. tumefaciens* strain EHA101 from glycerol inoculums. Cultures were grown to different ODs to determine transformation efficiencies at each OD. *E. coli* cultures were grown to 0.2 OD and 0.6 OD and *A. tumefaciens* were grown to 0.2 OD and 0.4 OD. The *E. coli* strains were transformed with the plasmid puc19 and the *A. tumefaciens* strain was transformed with the plasmid ptf101.1.

Electrocompotent cells were prepared for electroporation for each strain and OD. *E. coli* cultures were prepared by adding pre-culture (12 mL for Dh5α and 10 mL for the more quickly growing $CcdB^R$) to 300 mL of fresh media (LB or 2xTY) and were grown at 37°C at 250 rpm, checking the OD of the culture around the time predicted from the growth curve determined earlier. Once the culture reached the desired OD, 150 mL of culture were transferred into a centrifuge tube and immediately put on ice for at least 2 hours to halt growth. From this point, cultures were not allowed to warm to above 4°C until they were used for electroporation.

Cultures were then centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded and the pellet was resuspended in 10 mL of wash solution by pipetting up and down (wash solution is 10% glycerol and 1/1000 HEPES). The culture was then transferred to a 50 mL Falcon screw-top tube and centrifuged at 3000 rpm for 6 minutes. The supernatant was discarded and the pellet was resuspended in 10 mL of wash solution by inversion, not by pipetting up and down, which could damage the cells. The culture was again centrifuged at 3000 rpm for 6 minutes and again the supernatant was discarded and the pellet was resuspended by inversion in 10 mL wash solution. After another 6 minute centrifugation at 3000 rpm, the pellet was resuspended by inversion in 5 mL wash solution and left on ice for 15 minutes. The culture was again centrifuged for 6 minutes at 3000 rpm and the supernatant was discarded. The pellet was then resuspended by pipetting up and down with 300 μL wash solution to make a solution of about 500 μL. 50 μL aliquots were transferred to 1.5 mL tubes and flash frozen in liquid nitrogen, then stored in the -80°C freezer overnight.

To electroporate the cultures, tubes were retrieved from the -80°C freezer and put on ice. Plasmid was added to the culture (2 μL of 10 pg/μL puc19 for *E. coli* and 1 μL of 10 ng/μL ptf 101.1 for *A. tumefaciens*) and the plasmid-culture mix was transferred to a pre-chilled 2mm electroporation cuvette. The cuvette was then put into the electroporator and electroporated using setting EC2 to pass a 2.49 kV current through the culture for 5.90 ms. If an arc occurred due to a high salt content of the culture, the culture was discarded. If no arc occurred, 250 μL of SOC liquid medium was added to the cuvette and the resulting 300 μL mixture was transferred to a 1.5 mL tube and incubated. *E. coli* was incubated at 37°C for 45 minutes and *A. tumefaciens* was incubated at 28°C for 2

hours. *E. coli* cultures were then plated on a selective medium (LB or 2xTY) containing ampicilin so that only transformed bacteria could grow. *A. tumefaciens* cultures were then plated on a selective YEP medium containing chloramphenicol, kanamycin, and spectinomycin so that only transformed bacteria could grow. *E. coli* plates were incubated at 37°C and counted the next day. *A. tumefaciens* plates were incubated at 28°C and counted 2 days later.

Data was analyzed with Microsoft Excel to find average colonies per plate, standard deviation, and transformation efficiencies.

Results

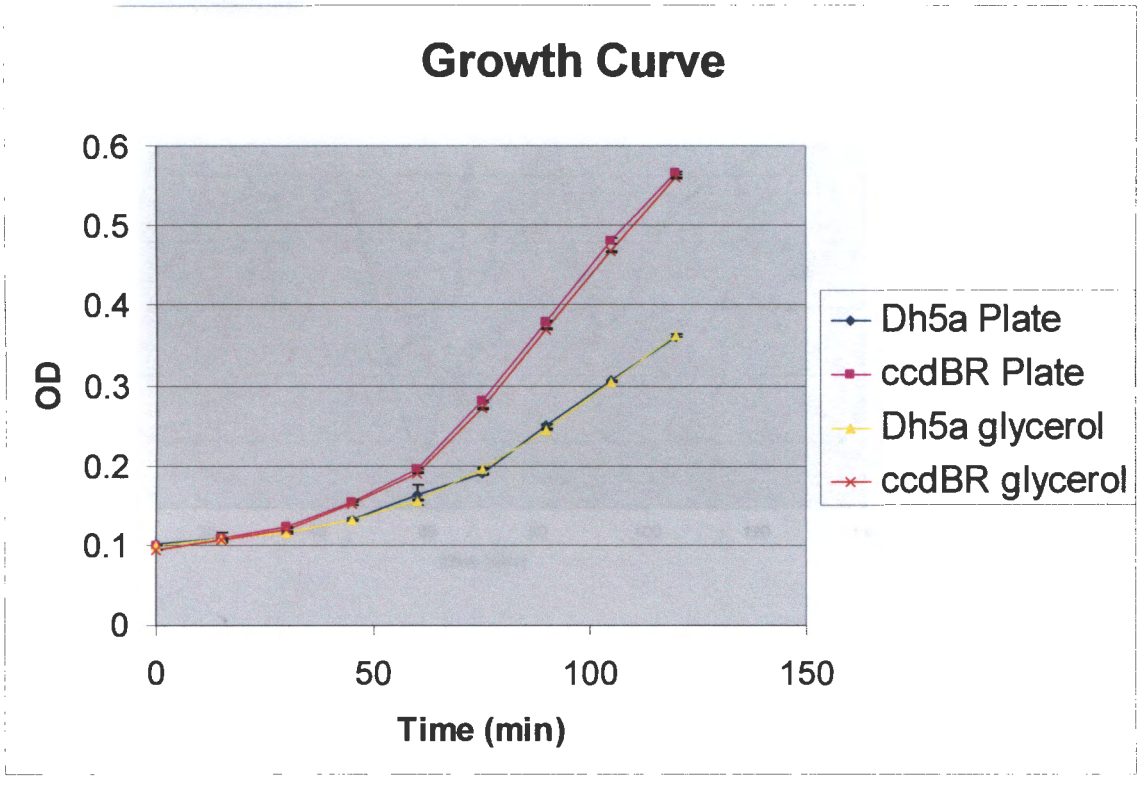

Figure 2: Optical Density Growth Data of *E. coli* grown in LB on 6-16-06

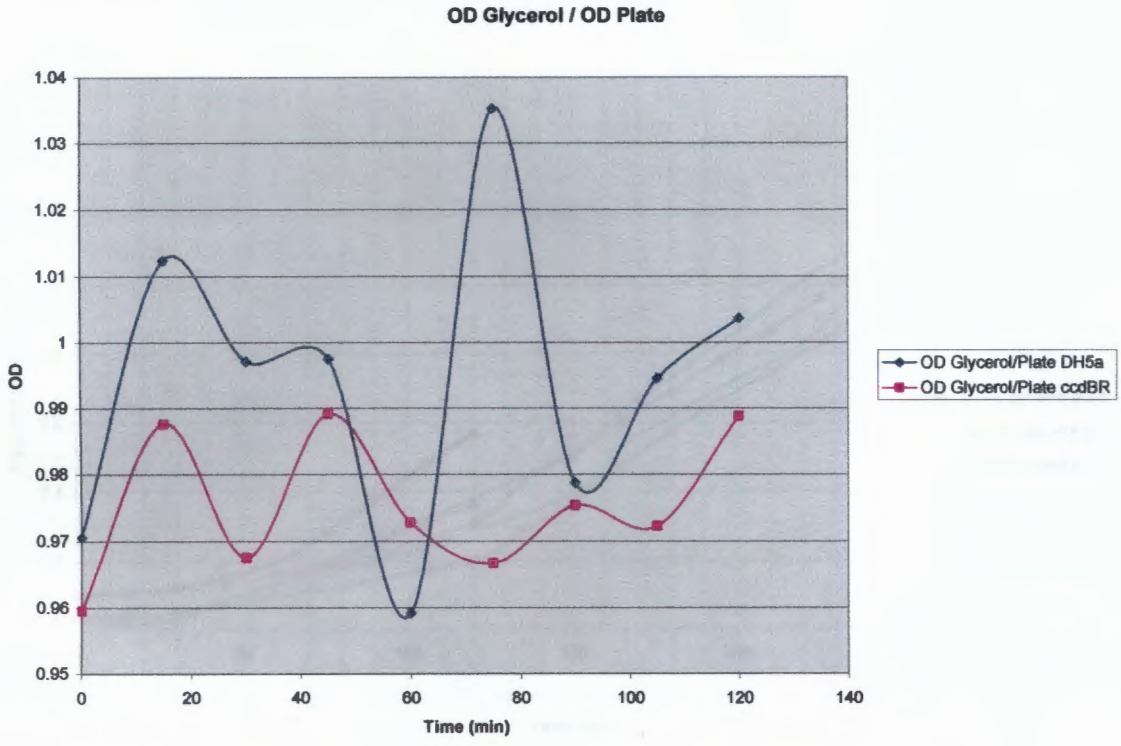

Figure 3: OD Glycerol / OD Plate Ratio for *E. coli* grown in LB on 6-16-06

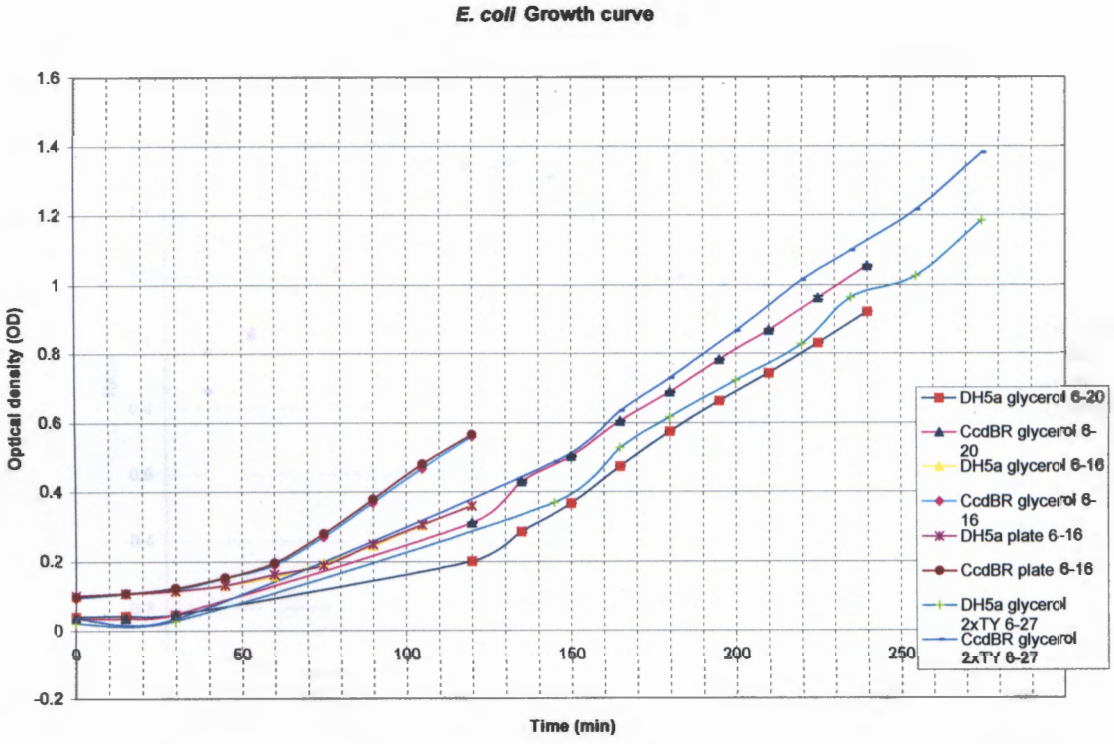

Figure 4: Optical Density Growth Data of *E. coli* grown from plate and glycerol inoculum in LB and 2xTY

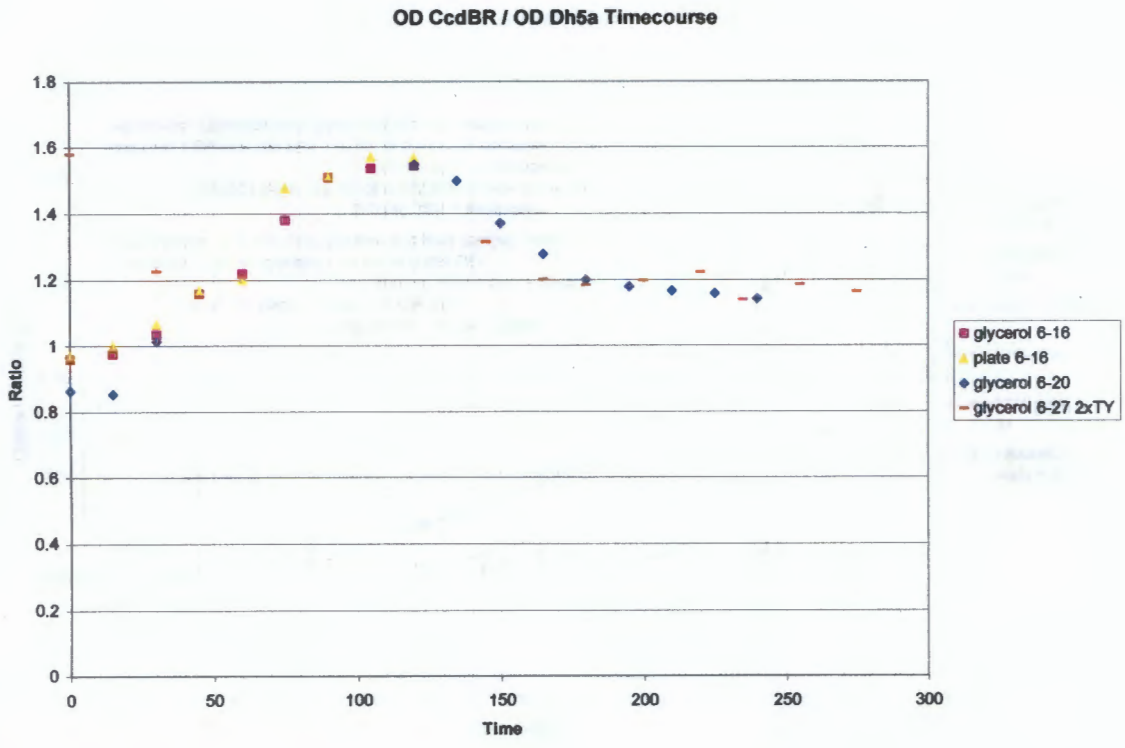

Figure 5: OD CcdBR / OD Dh5α Timecourse

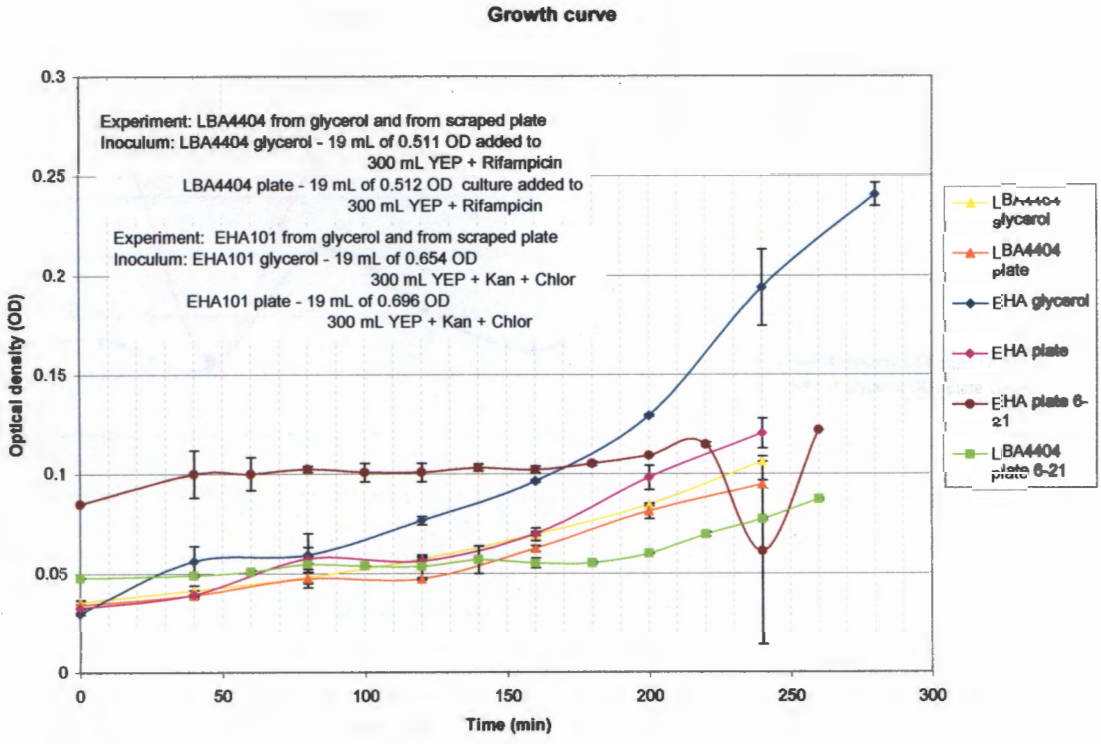

Figure 6: Optical Density Growth Data of *A. tumefaciens* grown from plate and glycerol inoculum in YEP + antibiotics.

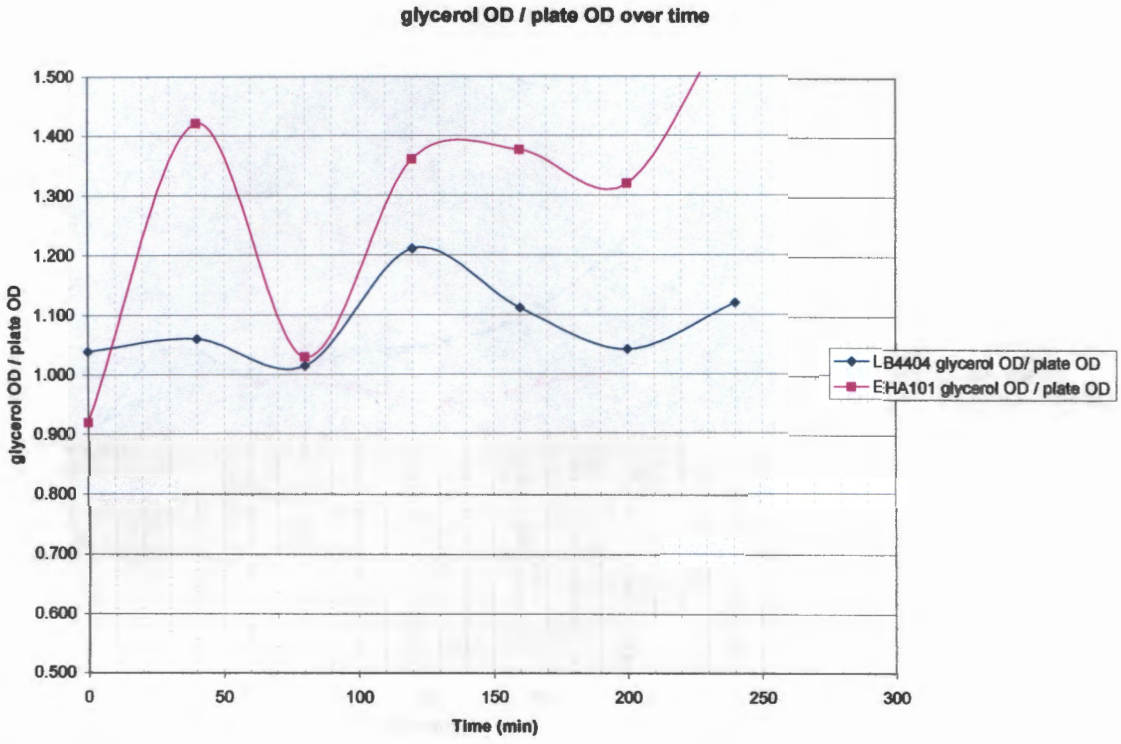

Figure 7: Glycerol OD / Plate OD Ratio Over Time for *A. tumefaciens* strains

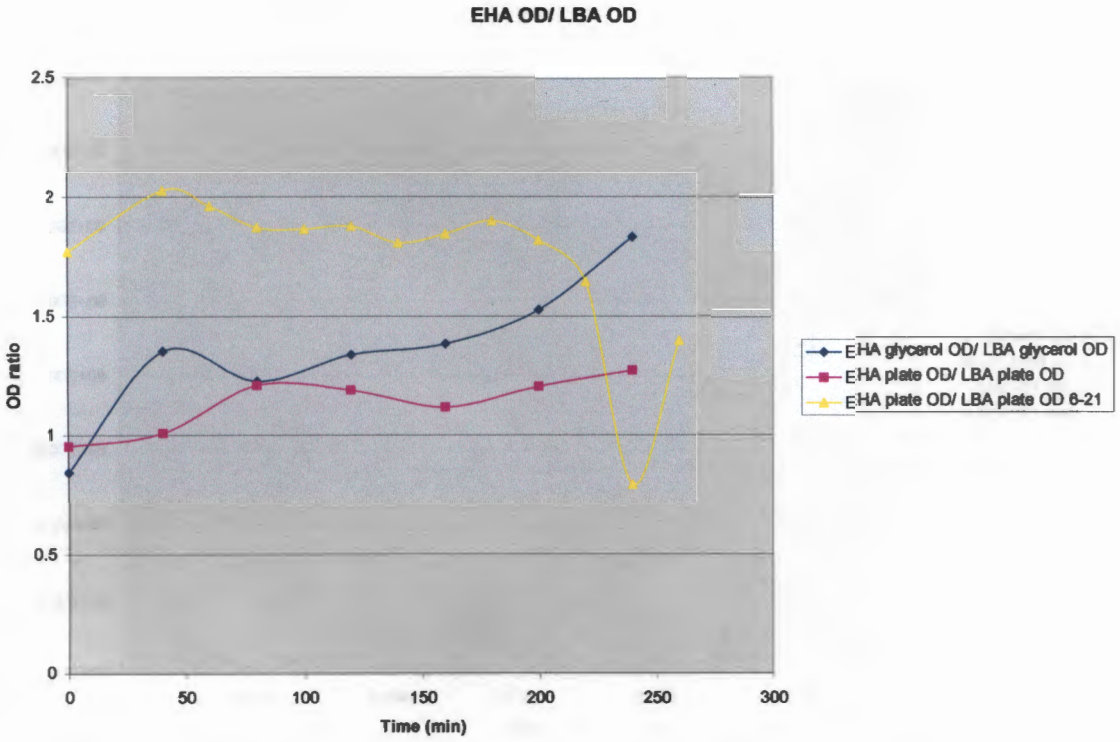

Figure 8: EHA OD / LBA4404 OD *A. tumefaciens* strains Ratio Over Time

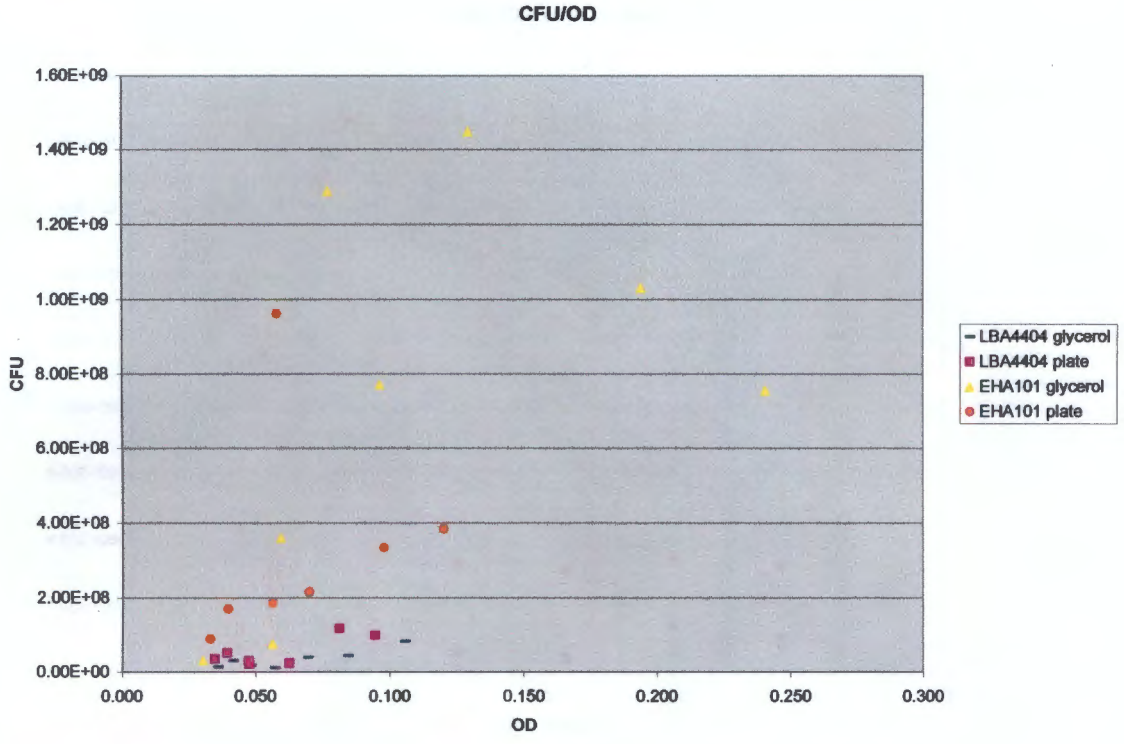

Figure 9 : CFU / OD Ratio for *A. tumefaciens*

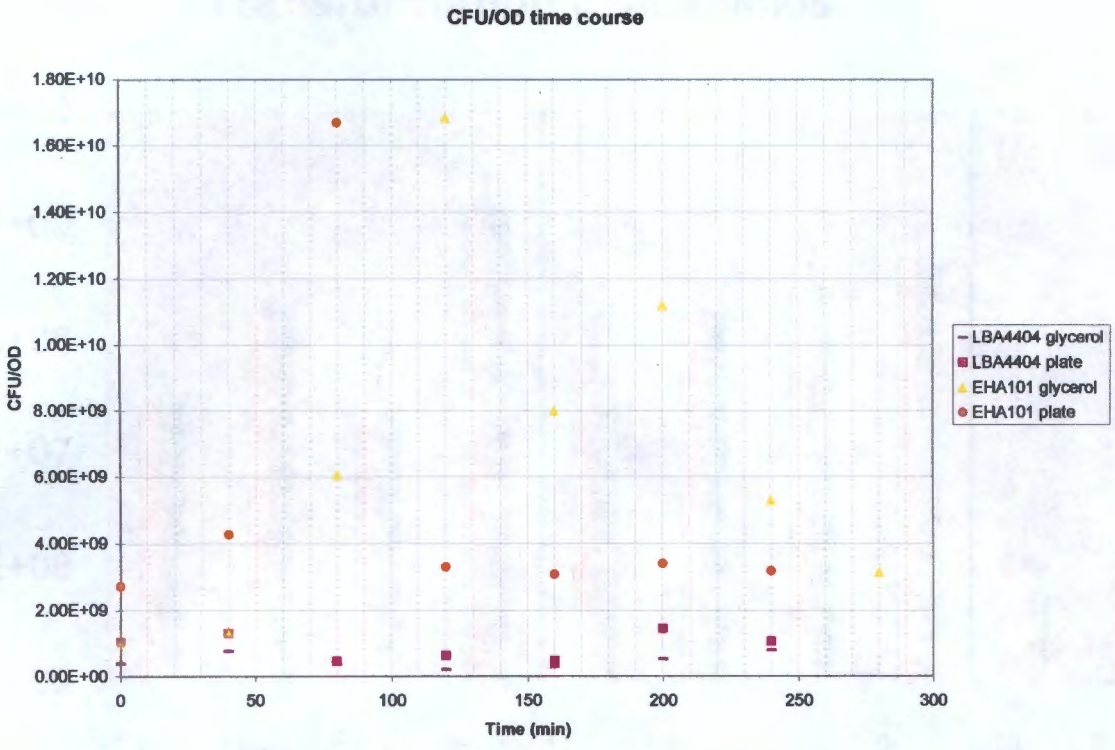

Figure 10: CFU / OD Ratio Over Time

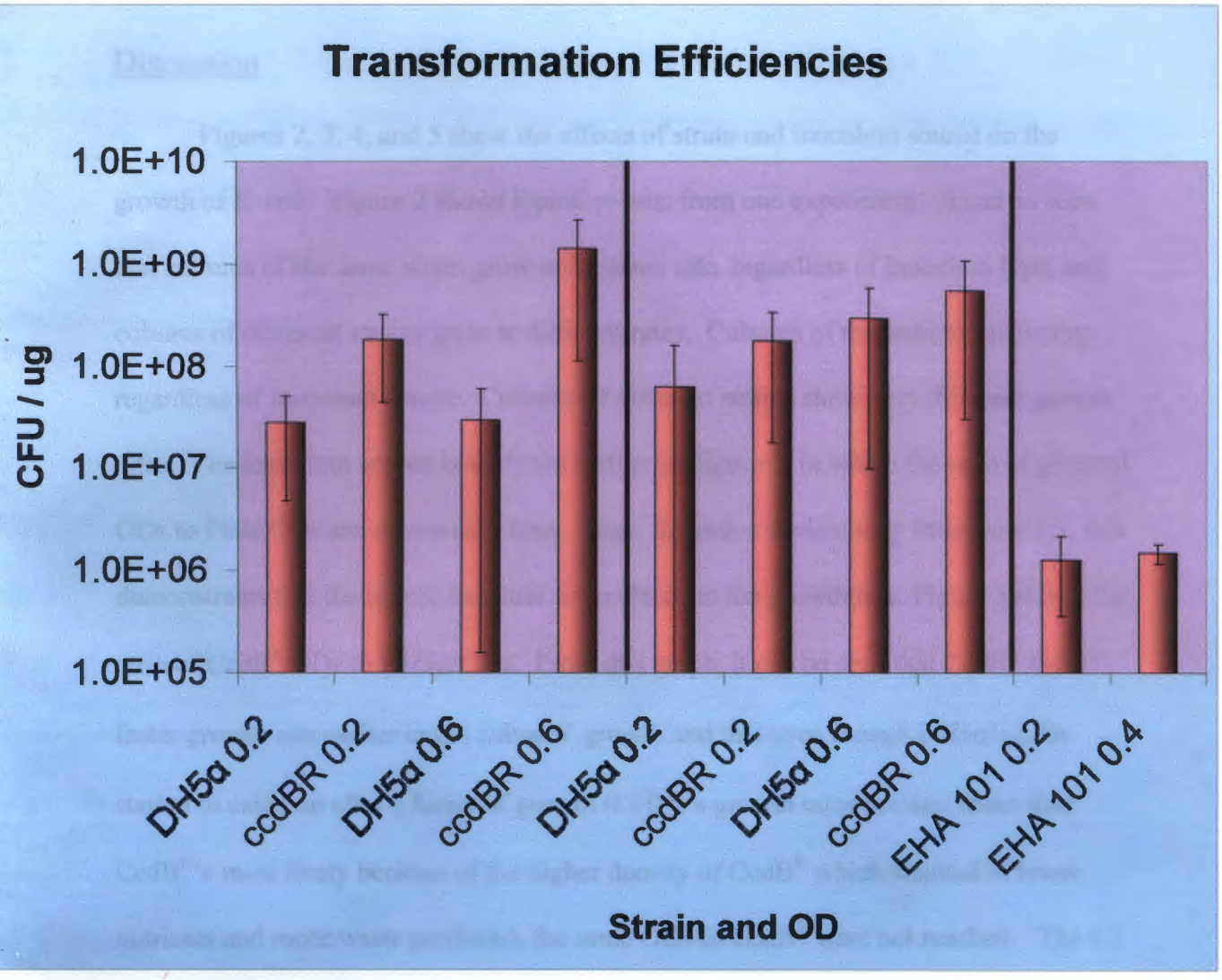

Figure 11: Transformation Efficiencies in cfu/microgram plasmid in *E. coli* and *A. tumefaciens* strains.

Discussion

Figures 2, 3, 4, and 5 show the effects of strain and inoculum source on the growth of *E. coli*. Figure 2 shows typical results from one experiment. It can be seen that cultures of the same strain grow at the same rate, regardless of inoculum type, and cultures of different strains grow at different rates. Cultures of the same stran overlap, regardless of inoculum source. Cultures of different strains show very different growth rates. The inoculum source is analyzed further in Figure 3, in which the ratio of glycerol ODs to Plate ODs are shown over time. Since the ratios deviate very little from 1:1, this demonstrates that the source has little to no effect on the growth rate. Figure 5 shows the ratio of $CcdB^R$ ODs to DH5α ODs. From this graph, it can be seen that $CcdB^R$ had a faster growth rate earlier in the cultures' growth and that even though DH5α's ODs started to catch up after 2 hours of growth (DH5α's growth rates became faster than $CcdB^R$'s most likely because of the higher density of $CcdB^R$ which resulted in lower nutrients and more waste products), the same ODs as $CcdB^R$ were not reached. The 0.2 to 0.6 OD range at 2 hours was later used for the transformation procedure.

Figures 6, 7, and 8 show the affects of strain and inoculum source on the growth of *A. tumefaciens*. Figure 6 shows compiled data for all experiments. Looking at this data more closely in Figure 7, which shows the ratio of glycerol inoculum ODs to plate inoculum ODs over time, the effect of inoculum type can be seen. Both strains deviated from a 1:1 ratio with glycerol inoculums having at least a slightly higher OD (growth rates fluctuated). This deviation was more pronounced in the EHA strain than it was in the LBA4404 strain. Figure 8 compares strains by showing the ratio of EHA OD to LBA4404 OD over time. In two of the experiments shown, strains were started at a 1:1

ratio and EHA showed a higher growth rate. For the 6-21 experiment, EHA was started at twice the OD that LBA4404 was started at. Despite the more crowded conditions (less nutrients per cell, more waste), the growth rates of the 2 strains stayed similar for the first 4 hours of growth.

Figures 9 and 10 show cfu to OD ratios for *A. tumefaciens*. The ratios stayed fairly level in most experiments, confirming that OD is a consistent indicator of cfu's. This was not seen in all experiments due to initial problems in the dilution procedure.

Figure 11 shows transformation efficiencies in cfu/microgram of plasmid in *E. coli* and *A. tumefaciens* strains. $CcdB^R$ showed higher efficiencies than Dh5α. The higher OD cultures had higher efficiencies. The media did not have a clear effect. The standard deviations are too large for these transformation efficiency results to be conclusive.

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