

Quantitation and Purification of Acquired Plasmid DNA Coding for Antimicrobial Drug Resistance

J. Roy Johnson Jr.

ABSTRACT

This study provides evidence of direct transfer of functional DNA from species to species, specifically *Escherichia coli* pGFPuv and *Enterobacter aerogenes*. An *Escherichia coli* made pGFPuv invasive by transformation via mini-prep transfers DNA after simple co-incubation with *Enterobacter aerogenes*. Transfer efficiency was enhanced or disturbed in the bacteria by altering pH. Expression of the acquired antibiotic resistant gene was detected in the recipient *Enterobacter aerogenes*, and the number of ampicillin resistant was quantitated and statistically analyzed to show transfer rates of the 1 kb gene. This procedure demonstrates a dire need for increase molecular investigations into the antimicrobial resistance that many pathogenic invaders are acquiring.

Keywords: *Escherichia coli*, *Enterobacter aerogenes*, pGFPuv, Conjugation, DNA purification, gel electrophoresis, Southern Blot

INTRODUCTION

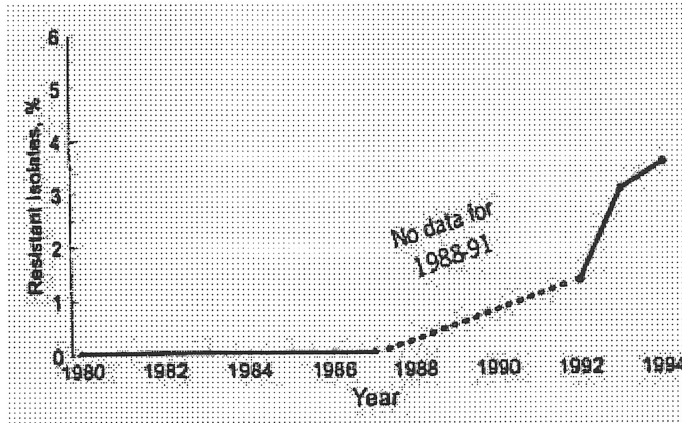
Horizontal gene transfer between bacteria is a common process, leading to the distribution of many traits, such as antibiotic resistance. Transfer occurs by several mechanisms like plasmid conjugation, (conjugative) transposition, bacteriophage transduction, and transformation (Bertram 1991, Frost 1992, and Lorenz 1994).

The efficiency and frequency of gene transfer depends on many variables, especially selective pressures. Gene transfer also depends on independent strains including their individual characteristics for genetic transfer. In addition to these variables and parameters, the various environmental parameters play a significant role in plasmid transfer who is the mechanism for gene transfer via conjugation between bacteria in natural environments such as colons, feces, tainted beef, or waster storage facilities. Until recently it was thought that antibiotic gene transfer via conjugation only occurred between closely related bacteria (Amabile-Cuevas 1992, Mazodier 1991). This study concentrates its efforts on horizontal antibiotic resistant gene transfer between two distant but distinct species of bacteria which occupy similar environments - such as intestinal tracts of humans and animals (Doucet-Populaire 1992). The bacteria *Escherichia coli* pGFPuv and *Enterobacter aerogenes* were selected for this study. The *Escherichia coli* plasmid was initiated with a green fluorescent protein (GFP) that is detectable with exposure to ultraviolet radiation (UV) just upstream of the antibiotic resistant gene.

One thing that has received little attention is the role of selective pressures within the organisms environments. Selective constraints alone do not stimulate gene transfer (except probably in the regulation of conjugative transposition in Bacteriodes

(Salyers 1995)), but they determine whether the organisms receiving new genetic material via conjugative plasmid transfer will reproduce and grow. For example, in this study, both organisms are intestinal flora, which are subjected to varying pH. pH allows a wide margin of selective pressure and reproduction is not restrained easily. According to the CDC, about three-quarters of outpatient antimicrobial use is for only 5 respiratory diagnoses. Therefore, if the doctors are treating a respiratory illness with a pill, then that agent passes through the digestive tract exposing the intestinal flora to the antimicrobial agent. If bacteria are reproducing and conjugating rather easily due to relaxed selective pressures, then once the resistance occurs in one organism, it will be passed around somewhat easily by conjugation. This example is how the problem starts, the solution needs immediate attention. This can be seen in the illustration below. Starting in 1992 there have been astronomical increases in antibiotic resistance, and as this chart shows, the limits have not been realized as of yet.

The human colon contains generations of different species of microbes, specifically bacteria. Two bacteria manifested in fecal material are *Escherichia coli* and *Enterobacter aerogenes*. Obviously as ubiquitous as bacteria are, these two organisms are found in farm animals, streams, rivers, and undercooked meat. Until three decades ago, antibiotics in human and animals without secondary consequences controlled bacterial growth. However, in the 1990's, bacteria are incubators for genes that produce certain proteins that resist antibiotic drugs whether the antibiotic is to destroy the cell wall, effect the ribosomes, or bind to the transfer RNA. Antibiotic resistance can evolve



CDC Figure. Rate of high-level penicillin resistance among 6721 invasive isolates of *Streptococcus pneumoniae*, United States, 1980 to 1994. Data from this CDC surveillance system were not reported from 1988 to 1991. (From: Butler JC, Hofmann J, Cetron MS, Elliott JA, Facklam RR, Breiman RF. The continued emergence of drug-resistant *Streptococcus pneumoniae* in the United States: an update from the CDC Sentinel Surveillance System. *J Infect Dis.* 1996;174:986-9

from mutations in the DNA or can be introduced into a host by horizontal conjugation creating a transformation in the host DNA. Horizontal gene transfer occurs by plasmid movement from the carrier bacteria with the resistance gene to the unaffected host, via the pili, which attaches the two bacteria during conjugation. This study manipulates the variable of pH, and quantitates the rate of conjugation. The goal is to determine the conditions at which maximum and minimum plasmid transfer occurs. This is studied by using the GFPuv plasmid in *E. coli*. The pGFPuv vector information was determined from the GenBank Accession #U62636. The total pGFPuv plasmid is about 3.3 kilobase pairs long. The green fluorescent gene accounts for approximately 1 kilobase, and the resistance gene is about the same in length. These two genes together make up over half the plasmid. pGFPuv carries the "cycle 3" variant of GFP described by Cramer et al 1996. This gene was cloned between the two MCSs of the pUC19 derivative pPD16.43 (Fire et al. 1990). The GFPuv gene can be easily excised from pGFPuv.

Alternatively, the GFPuv coding sequence can be amplified by PCR. The GFPuv gene was inserted in the frame with lacZ initiation codon from pUC19, so that a B-galactosidase-GFPuv fusion protein is expressed from the lac promoter in *E. coli*. Note, however, that if the GFPuv coding sequence is excised using a restriction site in the 5' MCS, the resulting fragment will encode the native (i.e. non-fusion) GFPuv protein. The pUC19 backbone of pGFPuv provides a high copy number origin of replication and ampicillin resistance gene for propagation in *E. coli*.

These characteristics will be seen in the final product, the *Enterobacter aerogenes*, after conjugation. The GFPuv makes the antibiotic resistant gene illuminate, detectable under ultraviolet light. This is the only reason that the GFPuv is needed, to detect the

downstream resistant gene (where one goes the other goes also).

MATERIALS AND METHODS

The mechanisms and actions of conjugation between *Escherichia coli* pGFPuv and *Enterobacter aerogenes* must be a very rare studied process. Protocols and procedures were not available for reference, so the following procedures were developed during the process of studying these organisms.

Using the Bergey's Manual, the media and selection criteria was determined. Luria-Bertrant Medium (LB) was used as agar and broth for all actions involved with these organisms. The media was prepared as follows:

To 950 ml of dsH₂O, add 10g bacto tryptone, 5 g bacto yeast, and 10g NaCl. If agar is desired, 15g/L of bacto agar are added to the previous agents. One important detail is to adjust the pH of the agar / broth solution to 7.0 with 1 M NaOH or HCl (LB is mostly acidic with range of 5.5 to 6.0 as determined in this experiment).

There must be a selection process for the final isolation of the bacteria that needs to be observed for gene transfer (*Enterobacter aerogenes*). Again, the Bergey's Manual was useful for determining the selection criteria for *Enterobacter aerogenes* from *Escherichia coli*. The selection used in this experiment was Potassium Cyanide (KCN). *Enterobacter aerogenes* is able to propagate on KCN media whereas *Escherichia coli* growth is hindered. The Bergey's Manual suggested adding 1.5 ml of a .5% KCN solution per 100ml of broth/agar. However, this concentration was not successful in halting *Escherichia coli* growth in this experiment. Therefore, the appropriate concentration of KCN had to be determined. After several weeks of plating *E.coli* on KCN plates, increasing the concentration slowly in .5% increments, the concentration was determined as follows: add 1.5ml

of 1.5% solution per 100ml of broth/agar. Note: the concentration upto 2.0% were tried, but was not needed after realizing that the pH of the agar had not been adjusted to 7.0.

After the selection factors for the *E. aerogenes* were determined, the selection criterion was determined for *E. coli*. The *E. coli* used in this experiment was ampicillin resistant *Escherichia coli* with pGFPuv. Therefore, ampicillin was the selection factor for this experiment due to the antibiotic resistance trait. *Enterobacter aerogenes* is not resistant and is sensitive to this antimicrobial agent. Therefore, the inhibitory concentration had to be determined using small increased increments, plating *E. aerogenes* on each. The concentration of ampicillin was determined as 20 ul amp / ml agar of a 10mg / ml stock solution of ampicillin. This conversion equals 200 ug amp per milliliter agar.

Both bacteria were used in the selection determination so that concentrations were pre-determined before conjugation occurred. After conjugation occurs between *E.coli* and *E.aerogenes*, *E.aerogenes*, with the ampicillin resistant gene, will be selected for on LB + 1.5% KCN + 200 ug/ml AMP agar. *E. coli* should not grow due to the KCN, and only *E. aerogenes* with the resistance trait will grow on the AMP.

Once the previous selection processes were accomplished, the experiment was able to move forward. In LB broth, the pH was adjusted to 6.5, 7.0, 7.5, and 8.0 using Tris and Mes as buffers (this was done due to the fact that propagating bacteria can effect the pH of solution). Now, in four (4) tubes of each pH, 50 ul of 12 hour cultures of *E.coli* and *E.aerogenes* were placed in the tubes for conjugation. The bacteria were allowed exactly 24 hours for "mating", and then each tube was spread on LB + 1.5% KCN + AMP plates for selection and growth (Figure 1) after serial dilutions for quantification. The dilution was 10 ul conjugated bacteria per 990 ul LB broth (tube A). Into tube B, 100 ul of tube A was combined with 900 ul of LB broth. Then, 100 ul of tube B was placed into 900 ul of LB broth in tube C. This was a 10,000 fold dilution of the 24 hour cultures which helps in counting colonies for quantification. 100 ul of tube C was then spread plated onto LB + KCN + AMP agar and incubated at 37 degrees C for 24 hours.

Also, after 24 hours of conjugation, each tube was checked with litmus paper for any major variation in pH, none noted. Due to the fact that the *Enterobacter aerogenes* was resistant to ampicillin after conjugation, a 1% agarose gel electrophoresis was done to determine similar molecular weights of DNA (which simply will show matching genes in the original pGFPuv *E.coli* and the transformed *E.aerogenes* (see Figure 2 results for disturbing details). The *E. aerogenes* pGFPuv was retrieved from the LB + KCN + AMP spread plate via sterile toothpick and placed into LB broth for growth. This was done to retrieve the bacterial DNA from the organism. DNA purification was done using the protocols for DNA miniprep on bacterial

cells. And, the 1% agarose gel electrophoresis was done according to standard procedures for electrophoretic analysis.

Due to the results of the gel electrophoresis, a DNA identification will be done using the Southern Blot technique. Complementary DNA for the resistant gene was ordered with the vitamin (CoEnzyme) Biotin attached to the 5' end. The darker will be delivered by supplying antibodies that recognize biotin specifically.

On the antibody, an enzyme is transported to the site where once a substrate is supplied a product (label) attaches to the DNA in question and can be seen on the nitrocellulose paper confirming the desired sequence (results not in for this paper).

RESULTS

The final concentrations that were determined as selective for these organisms were very difficult to determine. One major thing that must be noted is that if the pH of the agar or broth is not adjusted to 7.0 then there are different results. Agar at pH 7.0 grows the following: on LB + KCN *Enterobacter aerogenes*, on LB + KCN + Amp no *Enterobacter aerogenes*, on LB + Amp *Escherichia coli* grows, and on LB + KCN + Amp *E. coli* does not grow. This is expected prior to the trials. However, if the pH is not adjusted to 7.0 in the original agar or broth, there is significant changes. The pH of the LB agar or broth is in the range of 5.5 to 6.0.

The acidic solution reacts with either KCN or Amp because the results are the same as those mentioned previously. However, on the LB + KCN + Amp, *Enterobacter aerogenes* is able to grow very efficiently. This should not be possible due to the Ampicillin in the agar.

This would lead us to say that *the Enterobacter aerogenes* is naturally antibiotic resistant which is not the case, because resistance of the original strand was checked prior to conjugation. One other major finding was that after the pH was adjusted to 7.0 prior to inoculation, the concentration of KCN was lowered to 1.5% from 2.0%.

After conjugation occurred at each separate pH, the bacteria were spread on plates and allowed to grow for 24 hours. Three plates at each pH were then quantified using a random selection process and the plate count technique. There was 17 possible squares in the counter to select from, so for each plate each square was assigned a number. For each plate, a random number was selected which determined the box that was counted. The results were interesting, deriving from each group the mean and standard deviation (Figure 1).

There were still questions about confirming this DNA transfer; therefore, a 1% agarose gel electrophoresis was run using purified DNA from controls, ladders, and the DNA in question. Alarming results occurred, comparing the original *E.coli* pGFPuv to the lane with the confirmed *Enterobacter aerogenes* with ampicillin resistant DNA, there was no similarities between molecule sizes. Several gels was run, one using

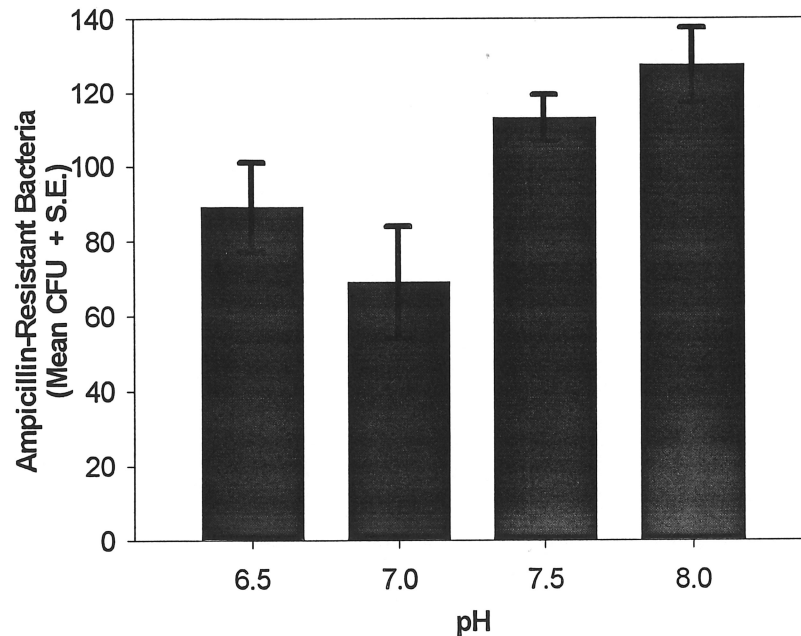


Figure 1. Acquired Ampicillin Resistance in *Enterobacter aerogenes* per group of varied pH.

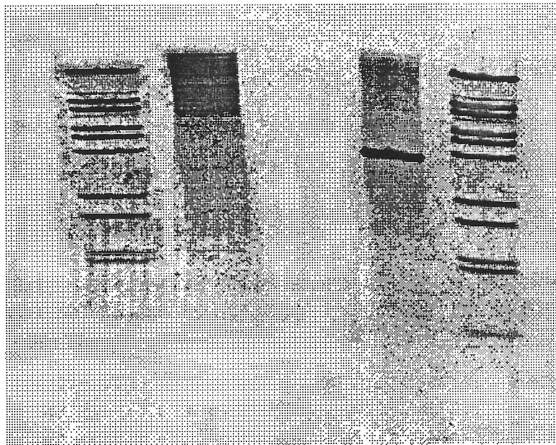


Figure 2. 1% Agarose Gel Electrophoresis Results. Lane 1 and 5 represents a ladder which are known molecular weights. Lane 2 is transformed *Enterobacter aerogenes* DNA, and Lane 4 is *E. coli* pGFPuv. Lane 3 is the original *E. aerogenes*.

restriction enzymes Hind III, Pst I, and EcoRI, and the same data came forth (Figure 2).

Due to the fact that the transformed bacteria is antibiotic resistant and did not show any characteristics to the *E. coli* DNA, as seen in Fig. 2, a Southern Blot of the purified DNA should be run to determine if the actual sequence for the antibiotic resistance was incorporated into the DNA of the *Enterobacter aerogenes*.

DISCUSSION

This topic is a prime concern of many agencies in America including the CDC. However, I was not able to find any research done on these two bacteria. Therefore, it is difficult to reference the findings of this project with another project.

As seen in Table 1, the results of conjugation did not proceed the way it was originally perceived. As Figure 1 demonstrates, transconjugal bacteria (especially *E. aerogenes*) propagate proportionally to the increase in pH. There is one outlier, pH 7.0. However, it is believed, if the total colony count increased, then even this pH would concur with the growth pattern. This demonstrates the answer of the original question of how pH affects conjugation. The Figure distinctly shows that conjugation rate increases; however, in finding this answer many more questions arose.

It was originally thought that the transformed *Enterobacter aerogenes* would illuminate the green fluorescence under ultraviolet radiation as the original *E. coli*. However, that did not occur, and yet, the *E. aerogenes* became resistant to ampicillin after conjugation. There is one possible answer to this unanswered question. The cytoplasmic enzyme, Rec A, degraded the plasmid after conjugation and then ligated the plasmid DNA with the host DNA, making it recombinant in the host DNA. This still shows that the host DNA was transformed.

This idea had to be shown worthy, so a Southern Blot of the DNA should be performed. This should

indicate the gene that was recombined into the genome using cDNA and a label.

The 1% agarose gel electrophoresis provided the evidence that the plasmid was not intact within the *Enterobacter aerogenes*. There were no matching bands from the original bacteria and the transformed bacteria. As seen in Figure 2, lane 4 *E. coli* pGFPuv (left to right) indicates a dark band, that is the plasmid pGFPuv. If that is compared to lane 2 ampicillin resistant *E. aerogenes*, there is not a matching band.

Therefore, *E. aerogenes* became resistant without demonstrating that it has the pGFPuv plasmid. This is why RecA enzyme was suggested as the probable cause. That enzyme could break down the plasmid DNA, and recombine it in the host DNA, whereas, there would not be a plasmid band in lane 2. Lane 1 and 5 are ladders to determine probable molecular weights of the bands. Lane 3 (clear) was the original *E. aerogenes*, and it has no plasmid as indicated.

Finally, this project has opened up many doors for future research. The questions and answers are not fully satisfied in my mind. For instance, why does the conjugation rate increase with pH? How does RecA effect the transconjugal plasmid? And, how was the gene for antibiotic resistance incorporated into the host DNA, what are the homologs?

Surely, antibiotic resistance is a growing problem in the world. This has occurred from misuse and mis-handling of these antimicrobial wonder drugs. Future problems are occurring at the present, just as our problems began decades ago. The search for answers in the field of microbial resistance must increase if we, as humans, wish to stay ahead of the pathogens that invade and live in our bodies.

ACKNOWLEDGEMENTS

I would like to personally thank Mr. Harry Stine for his gracious donations which made this research possible.

I would also like to thank my mentor, Dr. Jonathan Frye, for always answering my questions, and more importantly, motivating me constantly. And finally, I would like to thank Dr. Andy Bobb, who introduced and walked me through the difficult molecular biology techniques. He is a great addition to the Biology department.

LITERATURE CITED

- Amabile-Cuevas, C.F., and M.E. Chicurel. 1992. Bacterial plasmids and gene flux. *Cell*. 70:189-199
- Bertram, J., M. Stratz, and P. Durre. 1991. Natural transfer of conjugative transposon Tn916 between gram-positive and gram-negative bacteria. *J. Bacteriol.* 173:443-448
- Chalfie, M., et al. (1994) *Science*. 263:802-805.
- Cramer, A., et al. (1996) *Nature Biotechnol.* 14:315-319.
- Doucet-Populaire, F., P. Trieu-Cuot, A. Andremont, and P. Courvalin. 1992. Conjugal transfer of plasmid DNA from *Enterococcus faecalis* to

Escherichia coli in digestive tracts of gnotobiotic mice. *Antimicrobial Agents Chemother.* 36:502-504.

Frost, L.S. 1992. Bacterial conjugation—everybody's doing it. *Can. J. Microbiol.* 38:1091-1096.

Grabow WOK, Prozesky OW, Smith LS. 1974. Drug resistant coliforms call for review of water quality standards. Review paper. *Water Res.* 8:1-9.

Lorenz, M.G., and W. Wackernagel. 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol.* 53:665-671.

Mazodier, P., and J. Davies. 1991. Gene transfer between distantly related bacteria. *Annu. Rev. Genet.* 25:147-171.

Moellering, R.C. 1990. Interactions between antimicrobial consumption and selection of resistant bacterial strains. *Scand. J. Infect. Dis. Suppl.* 70:18-24

Nikolich, M.G. Hong, N. Shoemaker, & A.A. Salyers. 1994. Evidence that conjugal transfer of a tetracycline resistance gene (*tetQ*) has occurred very recently in nature between the normal microflora of animals and the normal microflora of humans. *App. Environ. Microbiol.* 60:3255-3260