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Transformation of a Mixed Probiotic Culture and *Escherichia coli B* with the Antibiotic Resistant Plasmid, *pGLO*

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ABSTRACT

Probiotics are microorganisms residing in the gastrointestinal tract that have been shown to help with metabolic functions, immune responses, and in the prevention of pathogenic diseases. In this study, we sought to determine if the probiotic brand, Garden of Life, could acquire novel phenotypes through heat-shock transformation. We predicted that Garden of Life, along with *Escherichia coli* HB101 and *Escherichia coli B* would be able to grow on ampicillin enriched plates after transformation with the plasmid, pGLO, which contained the gene for beta-lactamase. During the study, all three bacterial cultures underwent heat shock transformation in the presence of pGLO. After transformation was complete, each new culture was plated on LB agar containing ampicillin and arabinose to determine whether antibiotic resistance and GFP expression were observed. The results showed that only *Escherichia coli* HB101 had successfully become antibiotic resistant. Our results demonstrate that the gram positive species in Garden of Life probiotics are not able to efficiently take up plasmid DNA using heat shock transformation and that the method appeared to have strain restrictions as well.

1 INTRODUCTION

Probiotics contain specific species of living microorganisms that form a mutualistic relationship with the human digestive system. It is estimated that the microbiota present within and on the human body outnumber the human cells on a 10-fold scale (Turnbaugh et al., 2007) Studies that are currently being done have shown that bacterial diversity within the human gastrointestinal tract are greater than previously suspected (Eckburg et al., 2005). The different bacteria that make up the microbiota found in the human gastrointestinal tract express low to no pathogenic risks (Bengmark, 1998). Microorganisms play a significant role in our everyday lives and the consumption of probiotics can provide humans with an enriched microflora of these essential organisms.

Recently, there has been a growing interest in research regarding the benefits probiotics have on the human body. It has been found that probiotics improve immune functions and responses, as well as metabolic activities (Guarner & Malagelda, 2003). This can lead to nutrient exchange and absorption, as well as the instructing of host innate immunity (Sonnenburg et al., 2004). Metabolic activities such as obesity and weight management have also been recently linked to gut microbiota through gene sequencing of Christensenella minuta (Rosa et al., 2017). There is also a considerable amount of evidence to support the role of probiotics in the prevention of diseases. When probiotics colonize the large intestine, they may occupy all available epithelial and mucosal surfaces and thus prevent potentially pathogenic bacteria from colonizing the host. Additionally, pathogens still have to compete with the probiotics for nutrients. In cases where pathogens are able to grow, further expansion of the pathogen population is hindered by the presence of probiotics within that area (Rolfe, 2000).

Probiotics have been successful in preventing many gastrointestinal diseases such as Clostridium difficile, inflammatory bowel diseases, irritable bowel syndrome, cholera, and many other bacterial and viral infections (Rolfe, 2000). Due to the new found benefits of probiotics, sales have been increasing over recent years. A study found that between 2007 and 2012, probiotic sales had risen four times, thereby making it the third most expensive dietary supplement on the market (Clarke et al., 2015). The sales of probiotic enhanced foods have also increased over time and are now residing at a 1.4-billion Euro industry, which is equivalent to 1.5 billion in American dollars (Saxelin, 2008).

As probiotics are becoming more prevalent, antibiotic prescriptions are also increasing. Antibiotics disrupt the cellular physiology of bacteria, resulting in death of the microbial cell (Ding, 2010). Antibiotics are frequently prescribed to patients who experience viral infections, such as bronchitis, colds, and upper respiratory tract infections (URIs) (Nyquist et al., 1998). In ailments that are viral, antibiotics are misused and have no effect on the diseases (Nyquist et al., 1998). Studies have shown that antibiotics were prescribed up to 75% of time in patients diagnosed with the common cold, URIs, and bronchitis (Nyquist et al., 1998). The over-prescription of antibiotics makes pathogenic bacteria more resistant to treatment, as well as causes harm to the patients indigenous flora (Ding, 2010). When antibiotics disrupt indigenous flora, the beneficial bacteria that assist in bodily functions are inhibited, thus resulting in infectious diseases being able to colonize (Ding, 2010). This hindrance can often lead to a secondary infection within the patient (Ding, 2010).

Knowledge of probiotic benefits and antibiotic-caused damage has generated a need to restore and protect the indigenous flora within the human body. In this study, we sought to transform probiotic species through heat shock transformation with the pGLO plasmid. This plasmid contains the gene for green florescence protein, which was originally isolated from the jellyfish, *Aequorea victoria*. The pGLO plasmid also encodes a gene for antibiotic-resistance (Bassiri, 2011). This gene, bla, transcribes mRNA for beta-lactamase, the enzyme responsible for resistance against ampicillin (Bassiri, 2011). The brand of probiotic used, Garden of Life, contained eleven species of *Lactobacillus*, and five species of *Bifidobacterium*. The probiotic culture underwent the transformation process alongside the control strain, *Escherichia coli* HB101, and a second *Escherichia coli* strain, B. This study not only focused on probiotic transformation, but also on whether the transformation process had an effect of different strains of *Escherichia coli*. We hypothesized that the Garden of Life probiotic, *Escherichia coli* HB101, and *Escherichia coli* B would transform to become antibiotic resistant.

2 METHODS AND MATERIALS

Growth of Probiotics

Several different probiotic brand samples were grown in vitro using nutrient broth over the course of a week before transformation was done. Align probiotic samples and Garden of Life samples were put into separate nutrient broth vials and incubated for three days. The samples were then plated onto nutrient agar plates which were incubated for an additional two days.

Gram Staining of Bacteria

Nutrient agar plates containing the probiotic cultures were taken out of the incubator after two days and examined for growth. Clean slides were used to gram stain the Garden of Life probiotic sample which was examined under a microscope.

Preparation of Nutrient Agar and Plates

Transformation was carried out using the reagents contained in the pGLO Bacterial Transformation Kit (BIO RAD) according to the manufacturers instructions. Agar was prepared from 500 mL of deionized water and the LB nutrient agar packet. The mixture was then stirred and autoclaved for an hour and a half at 121 °C. Once the autoclave finished, the mixture was set to cool at 50 °C for 30 minutes.

Two plates were labeled as "LB Agar". The LB Agar plates were then poured and set aside at room temperature to solidify for one day.

Three ml of transformation fluid, $CaCl_2$, was added to a dehydrated ampicillin vial. The entire rehydrated vial was then added to the remaining agar in the flask. Five plates containing ampicillin and agar were poured, two labeled "LB/Amp pGLO" and three labeled "LB/Amp +pGLO". The plates were set aside at room temperature to solidify for one day.

Three mL of transformation fluid was added to a dehydrated arabinose vial. The entire rehydrated vial was then added to the rest of the agar in the flask. Five plates containing ampicillin and arabinose were poured, two labeled "LB/Amp/Ara pGLO" and three labeled "LB/Amp/Ara +pGLO". The plates were set aside at room temperature to solidify for one day.

Rehydration of *Escherichia coli* HB101 and Bacteria Plating

Two hundred fifty µL of transformation fluid was added to the lyophilized *Escherichia coli* HB101 vial. In order to ensure the resuspension of cells, the vial was left to sit for five minutes. The vial was then shaken and streaked onto the plate labeled "LB Agar pGLO E. coli". At that time, the nutrient broth containing the Garden of Life probiotics were also plated onto the "LB Agar pGLO G of L Probiotics" plate. Both plates containing the *Escherichia coli* HB101 and probiotics were incubated at 37 °C for 24 hours.

Preparation of pGLO Plasmid

Two hundred fifty µL of transformation fluid was added to the lyophilized pGLO plasmid DNA vial. The vial was shaken and allowed to settle before use.

Transformation of Bacteria

This procedure was adapted from BIO-RADs pGLO transformation protocol. Three micro test tubes were labeled "+pGLO" and three were labeled "-pGLO". The three "+pGLO" tubes were labeled separately for *Escherichia coli* HB101, *Escherichia coli* B (Carolina Biological), and Garden of Life probiotic sample. The same was done for the "-pGLO" tubes. Two hundred fifty µL of transformation fluid was transferred into each micro tube. All tubes were put on ice for five minutes. One large colony was transferred from each starter LB agar plate into its two respected tubes. Ten µL of pGLO plasmid DNA was added to each "+pGLO" tube and mixed. All tubes were incubated on ice for 10 additional minutes. All tubes were heat shocked in a 42 °C water bath for exactly 50 seconds and then rapidly transferred back on ice for two minutes. Two hundred fifty µL of nutrient broth was added to each tube and incubated at room temperature for 10 minutes. One hundred µL from each tube was then transferred onto its corresponding plates and spread around to ensure proper coverage. Plates were incubated at 37 °C for four full days before collecting results.

Growth Determination

All experimental plates were observed for growth and recorded for the amount present as either "+" or "-". Colonies resulting from transformed cells were then counted.

3 RESULTS

Two brands of probiotics, Garden of Life and Align, were plated on nutrient agar plates before transformation. However, only the Garden of Life brand showed growth and was used in the experiment. Table 1 shows the amount of growth on each of the three types of plates used. The LB Agar was used as the control plate and contained growth of untransformed organisms of *Escherichia coli* HB101, *Escherichia coli* B and the Garden of Life probiotic. The most growth was seen on this plate. The LB/Ampicillin/Arabinose and LB/Ampicillin plates were divided into two groups, those that were plated with the untransformed organisms (-pGLO) and those that had gone through the transformation procedure (+pGLO). The LB/Ampicillin/Arabinose (pGLO) displayed no growth for *Escherichia coli* HB101, no growth for *Escherichia coli* B, and no growth for the probiotic culture. The LB/Ampicillin/Arabinose (+pGLO) exhibited a medium amount of growth for *Escherichia coli* HB101, but no growth for *Escherichia coli* B or the Garden of Life probiotic. The LB/Ampicillin (pGLO) displayed no growth for *Escherichia coli* HB101, no growth for *Escherichia coli* B, and no growth for the Garden of Life probiotic. The LB/Ampicillin (+pGLO) exhibited a medium amount of growth for *Escherichia coli* HB101, but showed no signs of growth from *Escherichia coli* B or the Garden of Life probiotic. *Escherichia coli* B was not plated on LB/Ampicillin/Arabinose (-pGLO) or LB/Ampicillin (-pGLO), therefore results for those plates were not applicable.

Figures 1 and 2 demonstrate the antibiotic plates containing the organisms that underwent transformation. In Figure 1, the LB Agar control plates (-pGLO) showed substantial growth for Escherichia coli HB101 and the Garden of Life probiotic. The LB/Ampicillin (+pGLO) plate with Escherichia coli HB101 had 58 smaller colonies, while the LB/Ampicillin/Arabinose plate had 54 larger colonies (Figure 2). In each of the plates that displayed

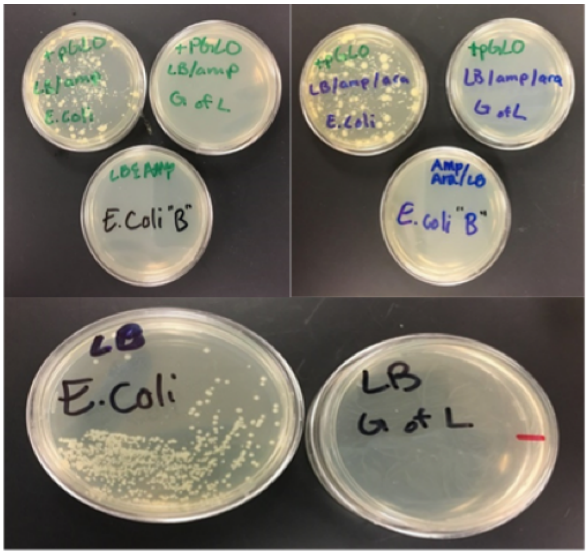

Fig. 1. Growth of Newly Transformed Organisms Containing the pGLO Plasmid on Antibiotic Infused Plates. The control plates, LB Agar on the bottom for comparison. Thr top left corner shows the LB/Ampicillin plates after the transformation. The top right corner shows the LB/Ampicillin/Arabinose plates after the transformation.

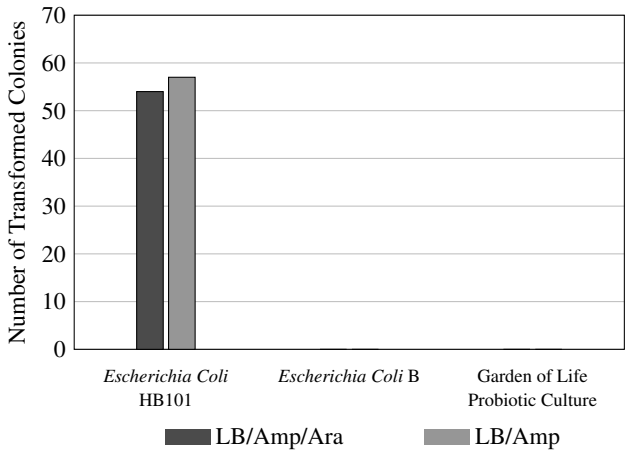

Fig. 2. number of Transformed Colonies Grown on Antibiotic Infused PLates "LB/Amp/Ara" and "LB/Amp". *Escherichia coli* HB 101 showed success of transformation as it had 54 transformed colonies growing on the LB/Amp/Ara plate, and 58 transformed colonies growing on the LB/Amp plate. *Escherichia coli* B had no growth on either antibiotic plate. Garden of Life Probiotics had no growth on either antibiotic plate.

growth after the transformation, a considerable amount of satellite colonies were observed (Figure 1). The smaller satellite colonies around the larger colonies on both the LB/Ampicillin and the LB/Ampicillin/Arabinose did not undergo transformation, as they were unable to express GFP, and were not counted in the final analysis (Figure 1).

4 DISCUSSION

Eleven species of *Lactobacillus* bacteria, five species of *Bifidobacterium*, and two strains of *Escherichia coli*, HB101 and B, were subjected to transformation process using the antibiotic resistant plasmid, pGLO. Out of the 18 viable bacteria specimens, the only one that successfully transformed was *Escherichia coli* HB101. The procedure for transformation used was the protocol from BIO-RADs Bacterial Transformation Kit, which was specifically designed for *Escherichia coli* HB101. *Escherichia coli* HB101 was therefore used as the control in our experiment in order to ensure that the transformation process was performed correctly. The *Escherichia coli* HB101 strain showed a slightly greater growth on the plate containing only LB/Ampicillin.

The satellite colonies were not counted when measuring the number of colonies on each of the ampicillin plates due to the fact that they did not undergo transformation. Satellite colonies are only able to grow on the plates due to a commensal relationship between the transformed and non-transformed *Escherichia coli* colonies (Smith et al., 2010). The transformed colonies secrete beta-lactamase which degrades the antibiotics on the plate thus creating an area that is free of ampicillin (Smith et al., 2010). This allowed for the satellite colonies to be able to grow on the ampicillin plates in the, now ampicillin-free, ring around the larger transformed colonies without having to be transformed (Smith et al., 2010).

The Garden of Life probiotic was used as our experimental culture. Figure 1 provides evidence that there was no growth found on either LB/Ampicillin/Arabinose or LB/Ampicillin plates, implying that the Garden of Life probiotic did not successfully undergo transformation to express antibiotic resistance. The

	***E.Coli* HB101**	***E.Coli* B**	**Probiotic Culture**
LB Agar (Control, -pGLO)	+++	+++	+++
LB/Ampicillin/Arabinose (+pGLO)	++	-	-
LB/Ampicillin/Arabinose (-pGLO)	-	N/A	-
LB/Ampicillin (+pGLO)	++	-	-
LB/Ampicillin (-pGLO)	-	N/A	-

Table 1. Growth of Organisms on Agar Plates. The amount of growth of *Escherichia coli* HB101, *Escherichia coli* B, and the probiotic culture are shown on all plates tested including LB Agar as the control. The "+" sign indicates the amount of growth on each plate. The "-" indicates no growth shown.

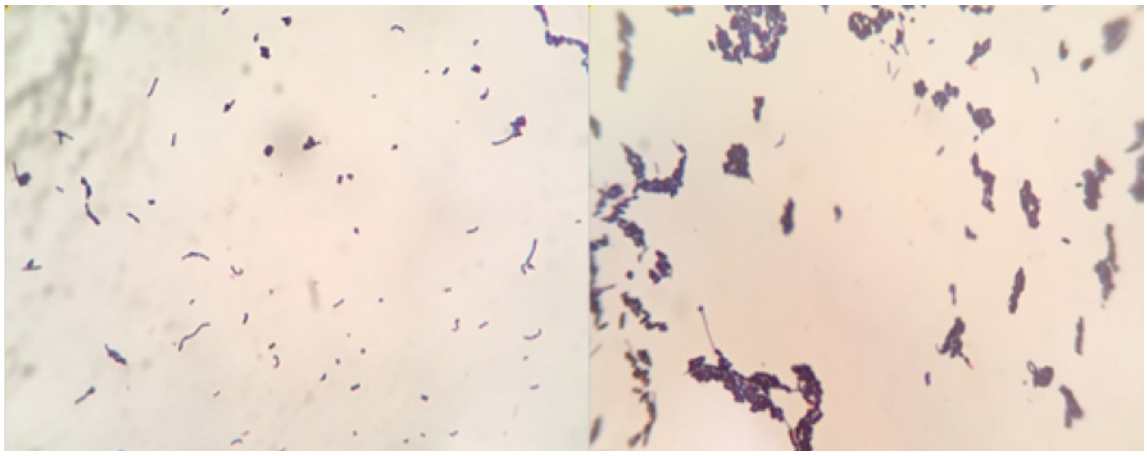

Fig. 3. Gram Stain of the Mixed Probiotic Culture Sample The probiotic mixture contained different species from the genera *Lactobacillus* and *Bifidobacterium*. Both genera are gram positive bacillus. *Lactobacillus* species are shown in thinner, smaller, and in a chain-like arrangements. *Bifidobacterium* species are shown in larger, thicker, and palisade arrangements.

probiotic sample used contained *Lactobacillus* and *Bifidobacterium* species that are all gram positive in nature, thus having a major physiological difference from our control, *Escherichia coli* HB101. Gram positives and gram negatives have been shown to take up DNA via the transformation method in ways that differ from each other. Gram positives take up DNA that bind in the peptidoglycan cell wall inversely from the ways that gram negatives do (Lorenz & Wackernagel, 1994). Gram positives also have a thicker cell wall that is relatively homogenous and lack an outer membrane. Gram negatives are fairly complex having several different layers, including a thin cell wall and an outer membrane containing lipopolysaccharides structures. The major ultrastructure differences seen between gram positives and gram negative signify permeability alterations (Schleifer & Kandler, 1972). We are inclined to believe that these are the major reasons as to why this transformation did not work on our gram positive probiotic culture. There are different methods of transformation such as electroporation that are often used that could potentially be successful for transforming these bacteria, as well as different ways to make bacteria chemically competent (Tenover et al., 1995). It is likely that these bacteria are transformable, but the method utilized was not efficient for inducing DNA uptake.

To begin the experiment we attempted to culture two different probiotic brands. However, only one of the samples actually grew when the probiotic was resuspended and plated on nutrient agar. Probiotics are fairly expensive in todays supplement market, and the lack of evidence regarding growth is concerning. This shows that not all probiotics on the market contain culturable bacteria even though they are advertised and ensured to contain a certain amount of viable cells within each dose.

Escherichia coli B was used to determine if different strains had an effect on transformation rates and success. When the LB/Ampicillin/Arabinose and LB/Ampicillin plates were analyzed, they showed that no growth had occurred (Figure 1). From these results, we can conclude that strain difference does have an effect on the success rate of transformation since *Escherichia coli* HB101 was successfully transformed but strain *Escherichia coli* B was not. Bacteria, much like multicellular organisms, have genetic differences that subdivide them amongst others of the same species (Dykhuizen & Green, 1991). In prokaryotes, this is referred to as a "strain" (Tenover et al., 1995). A strain can be defined as a member of one species that is distinguishable by phenotypic and genotypic characteristics (Tenover et al., 1995). Knowing that strain genetics differ amongst individuals within the same species, we have reason to believe that *Escherichia coli* B contains certain genetic traits that do not allow it to efficiently undergo transformation using the heat shock method. However, we were not able to perform genomic studies during this experiment therefore the exact trait variances cannot be differentiated. *Escherichia coli* B most likely lacks important traits necessary for this transformation that *Escherichia coli* HB101 has present within its genome.

Further research should be done to attempt the transformation of probiotics with exogenous genes. There are many other methods of transformation, as well as other ways to make bacteria competent that should be explored. These other methods could possibly yield our desired results to help society fix the negative impact that antibiotics have on the human indigenous flora, and potentially intersect the rising resistant pathogens.

The bacteria found in probiotics are species from the *Lactobacillus* genus, and species from the *Bifidobacterium* genus (Rolfe, 2000). *Lactobacillus* species have been found to benefit humans in many ways. There have been cases that show taking probiotics have an effect on lowering systolic and diastolic blood pressure in patients who suffer from high blood pressure (Robles-Vera et al. 2017). In other studies, multiple *Lactobacillus spp*. and *Bifidobacterium spp*. were shown to have an important role in protecting the gastrointestinal area against enteropathogenic infections (Inturri et al., 2016). We believe that there are more benefits to enhanced probiotics than disadvantages. With the ongoing issues in antibiotic-resistant "superbugs", there will be many future cases in which these probiotics would benefit human society and possibly inhibit certain diseases. Microbial antagonism,

using enhanced probiotics, could also be utilized as a new pathway for medicine in treatment against the antibiotic-resistant pathogens. Bacteria have a significant impact on the human body from being beneficial and necessary to pathogenic and fatal. The microbiota found within the human body help promote healthy immune systems, digestion, and overall bodily function (Eckburg et al., 2005). Scientists are just beginning to explore the ways in which probiotics enrich the diversity of microflora and their overall impact on the human body. The transformation of probiotics can potentially yield great medical advances for patients who lack healthy indigenous flora and in people that suffer from certain diseases. The accomplishment of probiotic transformation could potentially lead to more than just GFP or antibiotic-resistance gene expression in these bacteria. Transformation of probiotics with other genes that would be more beneficial, or enhanced genes of those already shown to have a positive impact on the human body, could also be exploited.

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