

The effects of the cancer metastasis promoting gene *CD151* in *E. coli*

Cara Burgess¹, Inez Yuwanita², Louise Pennycook², Youssef Ismail²

¹Peach Blossom School, San Jose, California

²Schmahl Science Workshops, San Jose, California

SUMMARY

The most feared and most deadly stage of cancer is stage 4, or metastasis. Metastasis is the ability of cancer cells to spread throughout the body. *CD151* is a known metastasis-promoting gene in humans. However, the independent effects of *CD151* in the process of metastasis are not known. We wanted to isolate *CD151* to discover what its role in metastasis would be on its own and uninfluenced by potential interactions with other components and pathways in human cells. To determine the independent effects of *CD151* on cellular motility and adhesion, we expressed *CD151* in bacteria and then performed several assays. Our results showed that *CD151* significantly increased the adhesion of the cells and decreased their motility. From this, we conjecture that *CD151* is upregulated in cancer cells for the last step of metastasis, and it increases the chances of success of metastasis by aiding in implantation of the cancer cells. Targeting *CD151* in chemotherapeutic modalities could therefore potentially slow or prevent metastasis.

INTRODUCTION

Cancer is a disease in which abnormal cells divide uncontrollably. It is the second-leading cause of death in the United States, causing 21.3% of total deaths per year, only slightly behind heart disease (1). According to the National Cancer Institute, approximately 39.5% of the population will be diagnosed with cancer sometime in their lifetimes (2). One of the most serious and life-threatening developments of cancer is metastasis. Metastasis is the ability of cancer cells to spread throughout the body. Cancer can spread locally to nearby tissue, lymph nodes, and organs, and even to distant parts of the body (3). Cancer metastasis kills more people than the original cancer itself, causing about 90% of cancer-related deaths (4).

To move through the body, cancer cells must break cell-cell junctions and detach from the original tumor. They then must enter the bloodstream or lymph system, which carries them away from the original site. Next, metastatic colonization may occur when cancer cells exit the bloodstream and implant in distant organs (3). While cancer cells often die during this process, some can form new tumors.

Certain genes enable cancer cell metastasis. These genes affect the adhesiveness and motility of cancer cells. We identified eight genes that could affect cancer cell motility:

CD151, *WASF3*, *CD44*, *TLR2*, *ROCK1*, *MMP2*, *MMP9*, and *NFKB1* (5-10). From the size of the coding regions of these genes, we narrowed down to three genes that would be small enough to insert into a plasmid: *CD151*, *TLR2*, and *MMP9*. In this project, *CD151* was tested because, unlike other genes, it affects both motility and adhesion (5). In this study, we tested the effects of *CD151* to determine whether it truly increased motility and adhesion. To test this, we inserted plasmids with the gene of interest into bacteria. Then, we compared the movement and morphology of these bacteria to other bacteria that did not have the gene.

Using bacteria allowed us to observe the independent effects of *CD151* itself on motility and adhesion. The previously observed effects of *CD151* on motility and adhesion could possibly have been due to interactions between *CD151* and different components and pathways in human cells. Because bacteria cells have completely different components and pathways than human cells, using *Escherichia coli* cells allowed us to see if *CD151* regulates motility and adhesion by itself.

We hypothesized that overexpression of *CD151* would affect the movement and morphology of the bacteria in such a way that it would promote the success of metastasis. Our research found that overexpression of *CD151* decreased motility but increased adhesion of the bacteria. Increased adhesion of cells would promote the success of the last stage of metastasis – implantation.

RESULTS

Four groups of bacteria were tested: untransformed bacteria (control group), bacteria transformed with a pUC plasmid (control group), bacteria transformed with a pGlo plasmid (control group), and bacteria transformed with a pGlo_CD151 plasmid (experimental group) (Figure 1). The pGlo plasmid contains a green fluorescent protein (GFP) gene, which fluoresces when bacteria are exposed to the correct wavelength of light. We used GFP expression to confirm that the pGlo plasmid and *CD151* were being expressed. *CD151* was expressed under an arabinose promoter so that it would only be expressed in the presence of arabinose in the growth media. A group of bacteria expressing the pUC plasmid was also included to check that our bacteria were capable of expressing a smaller plasmid. Since the pUC plasmid was smaller than the pGlo and pGlo_CD151 plasmids, if the pUC plasmid was expressed but the pGlo and pGlo_CD151 groups did not express their plasmids, then it would

suggest that the pGlo and pGlo_CD151 plasmids were too big to transform the bacteria with. Each type of plasmid also contained an ampicillin-resistant cassette, which allowed for selection of transformed bacteria by ampicillin resistance. Each group of bacteria was grown on three different agars: Luria-Bertani (LB), LB+Amp, and LB+Amp+Ara. The LB+Amp plates contained LB and ampicillin. The LB+Amp+Ara plates contained LB, ampicillin, and arabinose.

The transformation of the *E. coli* was successful as shown by the ampicillin resistance conferred to the transformed bacteria and the ability of the pGlo and pGlo_CD151 bacteria to glow under ultraviolet (UV) light. Five assays were performed on each of the groups of bacteria: optical density bacterial growth measurement, dot blot, agar motility, biofilm, and negative stain.

Optical density growth measurement

All groups of cells were grown for 24 hours starting from a standardized concentration, and then plated. The optical density of the cells was then measured. As expected, the optical density growth measurement indicated that untransformed cells were dead in LB+Amp and LB+Amp+Ara after 24 hours due to the presence of ampicillin (Figure 2). All other groups of cells stayed alive and grew after 24 hours. The group of cells that grew the least were the pGlo_CD151 in LB+Amp+Ara. This was also the only group where CD151 was expressed.

Dot blot

All groups of cells, besides the untransformed cells in LB+Amp and LB+Amp+Ara, expressed CD151 as shown by dot blot analysis (Figure 3).

Agar motility assay

All groups of cells were grown for 24 hours and then plated onto soft agar and incubated for 24 hours. All groups

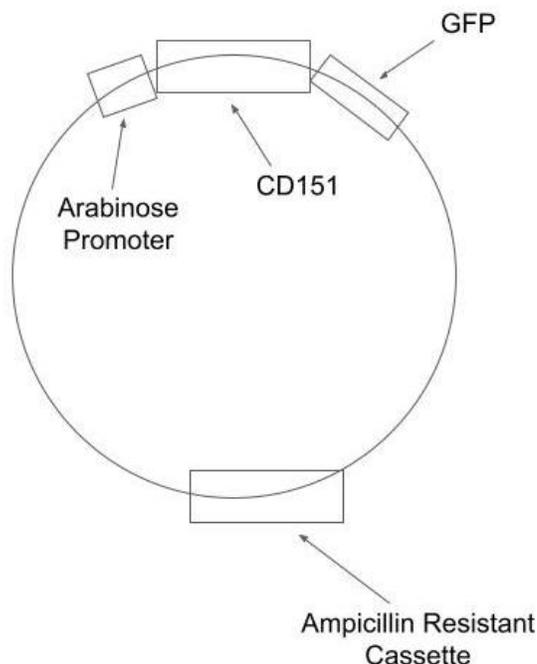


Figure 1. pGlo_CD151 plasmid. A representation of the pGlo_CD151 plasmid that the bacteria in the pGlo_CD151 group were transformed with containing an arabinose promoter, CD151, pGlo, and an Ampicillin Resistance Cassette.

of bacteria, besides the untransformed bacteria in LB+Amp and LB+Amp+Ara, had halos after 24 hours, meaning they had moved (Figure 4). The group that had the smallest halo, meaning that the bacteria were the least motile, was pGlo_CD151 in LB+Amp+Ara. This was the only group where CD151 was expressed. The pGlo_CD151 in LB+Amp+Ara group showed significantly less movement than the pUC in LB+Amp+Ara ($p = 0.0104$), pGlo in LB+Amp+Ara ($p < 0.001$), pGlo_CD151 in LB ($p < 0.0001$), and pGlo_CD151 in LB+Amp ($p < 0.001$).

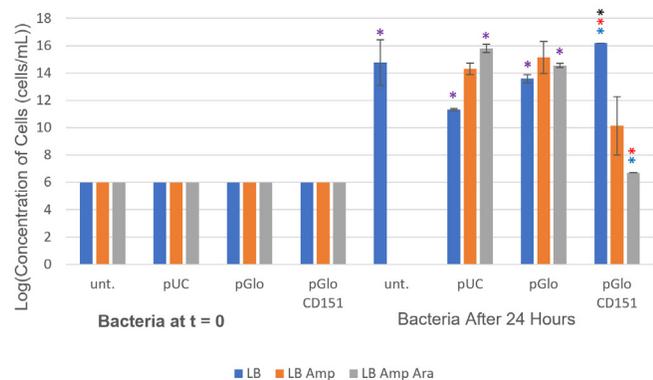


Figure 2. Concentration of cells before and after 24 hours growth. Each group had 3 replicates, and the error bars show the standard deviation of each group. The asterisks on the graph indicate whether the growth of a certain group was significant compared to another group (red = significant vs pUC; blue = significant vs pGlo; purple = significant vs pGlo_CD151). unt. = untransformed cells.

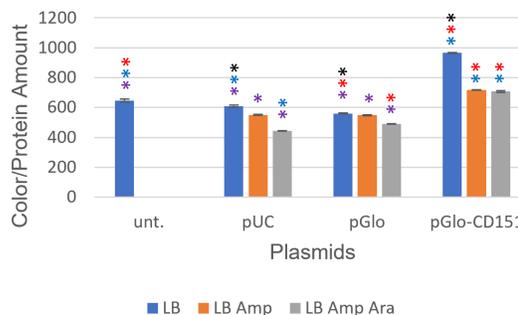


Figure 3. Protein amount after dot blot assay. Each group had 3 replicates, and the error bars show the standard deviation of each group. The protein amount, indicated by the color strength analyzed with ImageJ. The asterisks on the graph indicate whether the protein amount of a certain group was significant compared to another group (red = significant vs pUC; blue = significant vs pGlo; purple = significant vs pGlo_CD151). unt. = untransformed cells.

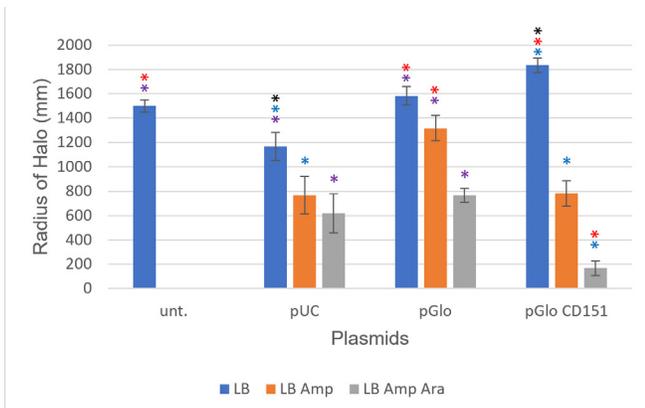


Figure 4. Radius of Halo after 24 hours – Trial 1 of agar motility assay. Each group had 3 replicates, and the error bars show the standard deviation of each group. A larger radius indicates greater motility. The asterisks on the graph indicate whether the motility of a certain group was significant compared to another group (red = significant vs pUC; blue = significant vs pGlo; purple = significant vs pGlo_CD151). unt. = untransformed cells.

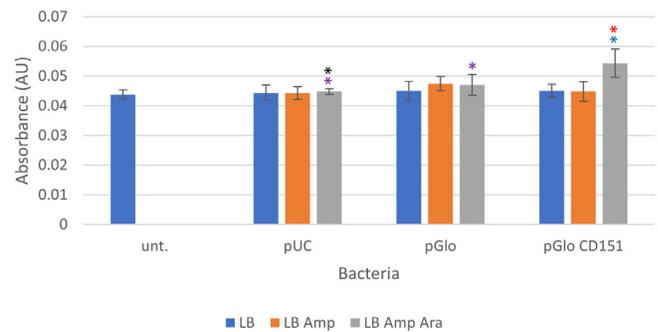


Figure 5. Absorbance of each well at 595 nm after biofilm. Each group had 4 replicates, and the error bars show the standard deviation of each group. A larger absorbance means the bacteria had greater adhesion. The asterisks on the graph indicate whether the adhesion of a certain group was significant compared to another group (red = significant vs pUC; blue = significant vs pGlo; purple = significant vs pGlo_CD151). unt. = untransformed cells.

Biofilm assay

The absorbance of each well was measured at 595 nm after formation of the biofilm. A higher absorbance means that the bacteria had a higher level of adhesion. The well with the highest absorbance pGlo_CD151 in LB+Amp+Ara, meaning that the bacteria in that group were the most adhesive (**Figure 5**). This was the only group where *CD151* was expressed. The pGlo_CD151 in LB+Amp+Ara group showed significantly more adhesion than the pUC in LB+Amp+Ara ($p < 0.01$), pGlo in LB+Amp+Ara ($p = 0.0493$), pGlo_CD151 in LB ($p = 0.0125$), and pGlo_CD151 in LB+Amp ($p = 0.0172$).

Negative stain

All groups of cells were stained with nigrosin and then viewed under a microscope at 100x in order to observe the morphology of the cells. After the negative stain, all groups of bacteria, besides the pGlo_CD151 in LB+Amp+Ara, displayed as individual bacteria cells under the microscope (**Figures 6 and 7**). However, the pGlo_CD151 bacteria in LB+Amp+Ara appeared as numerous long chains instead of individual cells. The pGlo_CD151 in LB+Amp+Ara group displaying the long chains of cells stuck together (**Figure 8**). This group was the only group where *CD151* was expressed.

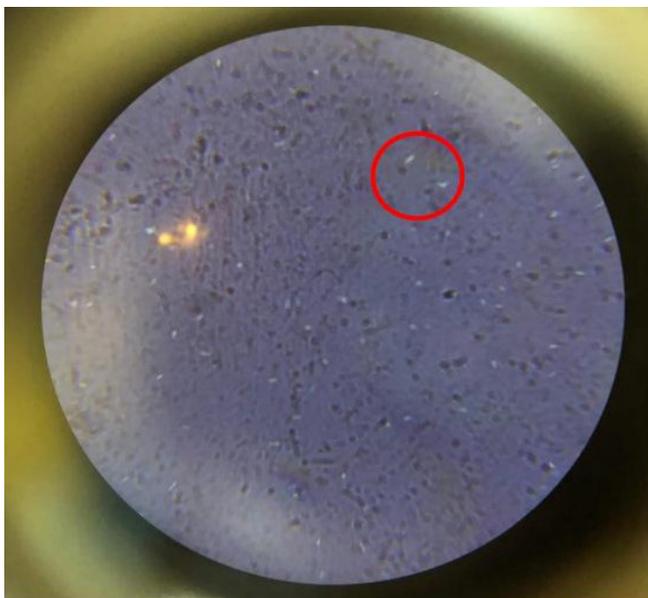


Figure 6. Untransformed bacteria in LB. Bacteria after the negative stain at 100x objective magnification. The individual bacteria cells can be seen in the red circle.

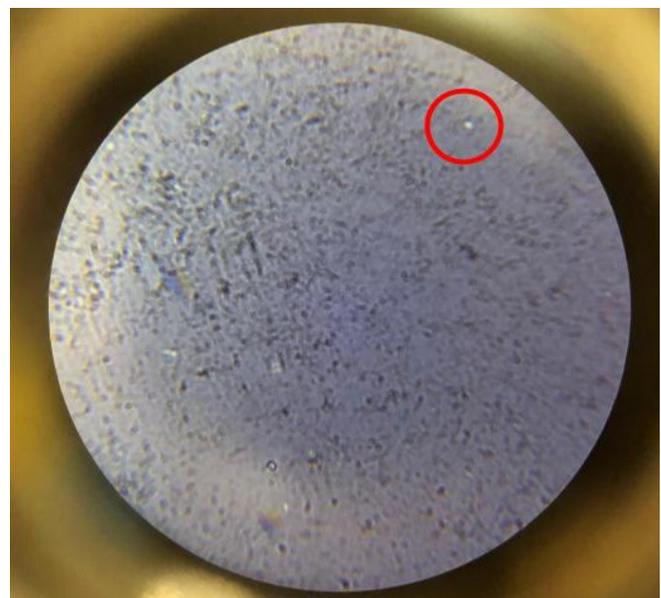


Figure 7. pGlo_CD151 bacteria in LB+Amp. Negative stain at 100x objective magnification. The individual bacteria cells can be seen in the red circle.

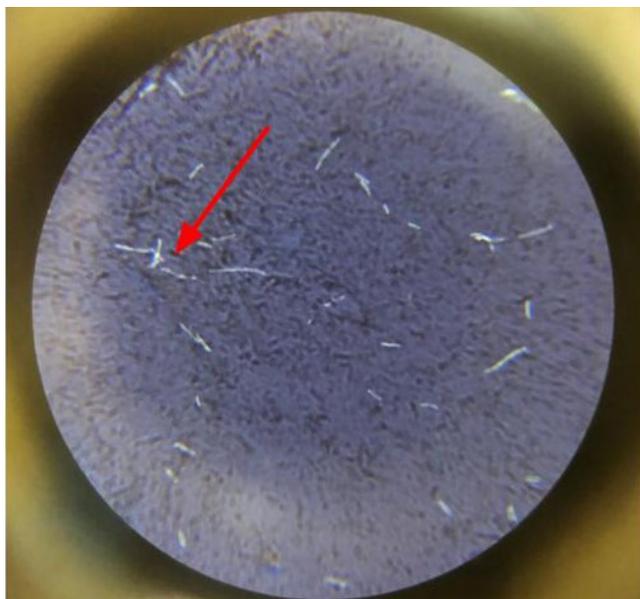


Figure 8. pGlo_CD151 bacteria in LB+Amp+Ara. Negative stain at 100x objective magnification. Because arabinose is present, CD151 is being expressed by the bacteria. The bacteria cells can be seen stuck together in long chains, as indicated by the red arrow.

DISCUSSION

Multiple tests showed that *CD151* increases the adhesion of bacteria cells. The results of the biofilm displayed that those bacteria expressing *CD151* had a significantly higher level of adhesion than bacteria that had the pGlo plasmid or bacteria that had the pGlo_CD151 plasmid but did not express *CD151* (i.e., not grown on media with arabinose).

In the negative stain, the bacteria expressing *CD151* appeared to be joined together in numerous long chains, while the *CD151*-non-expressing bacteria, as well as the untransformed, pUC, and pGlo bacteria showed up as individual cells or only a few short chains (**Figures 6-8**). The pGlo_CD151 bacteria in LB+Amp, where *CD151* is not expressed, displayed individual cells, while the pGlo_CD151 bacteria in LB+Amp+Ara, where *CD151* is expressed, displayed many chains of cells stuck together (**Figures 7 and 8**). Since the only difference between the pGlo_CD151 bacteria in LB+Amp and the pGlo_CD151 bacteria in LB+Amp+Ara is the expression of *CD151* in the bacteria, this difference in appearance must be the result of *CD151* expression. These results show that *CD151* not only increases the adhesion of bacteria cells to surfaces, such as the plastic used in the biofilm assay, but that it also strengthens the cell junctions and increases the adhesion of cells to each other, shown in the negative stain (**Figure 8**).

The motility assay showed that *CD151* also significantly decreases motility. Bacteria expressing *CD151* showed significantly less movement than bacteria with the pGlo plasmid or bacteria that had the pGlo_CD151 plasmid but were not expressing *CD151*. The observations made during the negative stain of the *CD151* expressing bacteria support

these results, as it would be much more difficult for the bacteria to move while stuck together in a long chain than it would be for individual cells to move. Therefore, our results suggest that upregulation of *CD151* increases the adhesion of the cell and thereby reduces cell motility.

As *CD151* is a human gene and not usually expressed in bacteria, a likely conclusion is that the effects observed are the direct effect of *CD151* and not the result of multiple genes or different pathways interacting. These findings indicate that *CD151* may aid metastasis by being upregulated in cancer cells for the last step of metastasis: implantation. When cancer cells have traveled to a different part of the body via blood vessels or the lymphatic system, they must implant in the tissue to grow and form a new tumor. The increased adhesion caused by the upregulation of *CD151* could help the cancer cells stick to the tissue and implant. This means that *CD151* could increase the chances of metastasis being successful and the cancer cells spreading.

A limitation of our study is that our research was done in *E. coli*, so the findings may not necessarily translate to human cells. Further research is needed to support our results and extend these findings to mammalian cells. Our findings clearly show how upregulation of *CD151* could potentially make it easier for metastasis to occur. Future research could focus on the effects of upregulation of *CD151* in mammalian cells, specifically how it helps cancer cells to metastasize, and the exact role the gene plays in the process.

MATERIALS AND METHODS

Bacterial transformation and culture

Four groups of bacteria were used in this study: untransformed bacteria (control group), bacteria transformed with a pUC plasmid (control group), bacteria transformed with a pGlo plasmid (control group), and bacteria transformed with a pGlo_CD151 plasmid that we designed (experimental group). We used DH5- α *E. coli* bacteria due to their strong ability in cloning plasmids. A DH5- α strain of *E. coli* was transformed with each plasmid via heat shock. The pGlo_CD151 plasmid included an arabinose promoter in front of *CD151*. This meant that *CD151* would only be expressed in the presence of arabinose. GFP was located after *CD151*, so that expression of the GFP, resulting in glowing bacteria, would indicate expression of *CD151*.

Three different mediums were used to grow the bacteria. Luria-Bertani (LB), LB with 100ug/mL ampicillin (LB+Amp), and LB with 100ug/mL ampicillin and 1% arabinose (LB+Amp+Ara). Each of the four groups of bacteria was grown in the three different mediums, except the untransformed bacteria. Since it did not have plasmids, and therefore was not resistant to ampicillin, it could not grow on the LB+Amp or LB+Amp+Ara plates.

Optical density bacterial growth measurement

A McFarland Standard (3.0×10^8 cells/mL) was made using LB broth and HB101 bacteria. Each of the four bacterial

groups were standardized to this McFarland Standard and all groups, including the standard were diluted 1:100. After 24 hours of growth at 37°C, 200µL of each culture was added to a 96-well plate. This was done three times for each tube. The optical density of each culture on the plate was measured at 600nm.

Dot blot

This protocol was adapted from Abcam (11). Briefly, 2 µL of the bacteria culture samples created during the Optical Density Bacterial Growth measurement (3.0×10^8 cells/mL) were spotted onto the nitrocellulose membrane at the center of the grid using a narrow-mouth pipette. The membrane was then left to dry. The membrane was blocked by incubating in 5% bovine serum albumin (BSA) in tris-buffered saline and polysorbate 20 (TBS-T) for 30-60min at room temperature (RT). The membrane was then incubated with a goat anti-rabbit IgG primary antibody (5 µg/mL in BSA/TBS-T; Sigma-Aldrich, Cat# A3687) for 30 minutes at RT. Then, the membrane was washed three times, 5 min each, with TBS-T and then incubated with a rabbit anti-CD151 secondary antibody (Cell Signaling, Cat# 17327) conjugated with HRP (horseradish peroxidase) for 30 min at RT. The membrane was then washed three times with TBS-T. Initially for 15 min, then twice for 5 min each. Next, the membrane was washed once with TBS for 5 min. Finally, the membranes were incubated with chromogenic reagent for 1 min then visualized. Images were analyzed with ImageJ.

Agar motility assay

This protocol was adapted from iGEM (12). Tryptone agar containing 0.25% agar and 100µg/mL ampicillin was prepared. Bacterial cultures were grown in LB broth at 37°C for 24 hours 1.5 µL of each sample was plated onto soft agar in the center of each plate. The plates were incubated for 24 hours at 37°C. After 24 hours, the plates were photographed.

Biofilm assay

The biofilm assay protocol was adapted from iGEM (13). Bacterial cultures in LB+Amp were grown for 18-20 hours at 37 °C in a shaking incubator. Dilutions of 1:100 were prepared for each liquid culture and the dilutions were incubated for 20 hours at 37 °C in a shaking incubator. Next, 100 µL of each dilution was plated in sets of 4 wells in a round-bottom 96-well plate. The 96-well plate was then covered and incubated at 37 °C for 48 hours. The plate was shaken out over a tray to remove all planktonic bacteria and rinsed in a large beaker of water. The water was then shaken out. The wells were then stained with 125 µL of 0.1% crystal violet for 10 minutes, and then the crystal violet was rinsed out. The plate was left face up to dry overnight. The next day, 200 µL of 30% acetic acid was added to all wells that were stained to solubilize the crystal violet. The acetic acid was left to sit for 10 minutes. The acetic acid/crystal violet mix was pipetted up and down to mix it in the wells, then 125 µL of the acetic acid/crystal

violet solution from each well was transferred into a well in a flat-bottom, 96-well plate. The OD595 of each well in the flat-bottom plate was read with a plate reader.

Negative stain

Staining protocol was adapted from Austincc (14). A single drop of nigrosine was placed on a clean microscope slide. Using a flamed loop and sterile technique, some bacteria was removed from the original sample and mixed into the drop of nigrosine. The end of another clean microscope slide was placed at an angle to the end of the slide containing the organism and moved to spread the drop out into a film. The film was allowed to air dry and was observed under a microscope.

Statistical analysis

The results of the assays were analyzed using a two-sample t-test using Microsoft Excel and a TI-84 Plus CE calculator. For each assay, the p-value for each group of bacteria (LB pGlo_CD151, LB+Amp pGlo_CD151, etc.) vs another group of bacteria was determined. The significance level was 0.05.

Received: July 27, 2021

Accepted: May 11, 2023

Published: June 11, 2023

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