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The Effects of Pretreatment of Competent Cells with Nalidixic Acid on Efficiency of Chemically-induced Transformation in *Escherichia coli* B23

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Transformation of *Escherichia coli* with foreign DNA is dependent on DNA binding and translocation at the cell surface. Nalidixic acid is an antibacterial agent that disrupts DNA replication and arrests the cell cycle, resulting in elongated, serpentine cell forms. This study investigated the effect of increased cell surface area, produced by exposure to nalidixic acid, on the transformation efficiency of *E. coli*. *E. coli* B23 cells were either treated with a sublethal dose of nalidixic acid or left untreated, then transformed with a plasmid encoding ampicillin resistance by using a calcium chloride/heat shock method. Cells treated with nalidixic acid had a transformation frequency of 5.6 x 10^{-10} cfu/µg per viable cell while untreated cells had a transformation frequency of 53 x 10^{-10} cfu/µg per viable cell. Our results demonstrate that cell elongation caused by nalidixic acid decreases transformation efficiency and suggest that this is due to lower viability following heat shock.

The uptake and incorporation of foreign DNA from the environment into bacterial genome is known as transformation (6). Bacterial cells capable of DNA uptake are called competent. *Escherichia coli* B23 is a gram-negative bacterium which is not naturally competent, hence it does not readily take up exogenous DNA from its environment (2). One method of artificially inducing a competent state is through treatment of these cells with calcium chloride, which increases cell membrane permeability (4). The mechanism of calcium chloride transformation of *E. coli* with plasmid DNA involves two important steps; one is the binding of DNA to the cell surface, and the second is the subsequent entry of DNA in to the cell cytosol triggered by a heat-shock from 0 to 42°C (6). Although the mechanism used for the uptake of plasmids in *E. coli* is still obscure, the proposed hypothesis is that DNA is taken up through zones of adhesion where the inner membrane and outer membrane fuse with pores in the cell wall (6). These zones of adhesion are rich in LPS, which are negatively charged, and the use of divalent cations in transformation forms a stable complex with the negatively charged backbone phosphates of DNA and the LPS, which facilitates the uptake of DNA (6). Given that the transformation process is a membrane bound phenomenon, this study investigated the possible effects of surface area on efficiency of transformation by pretreatment of *E. coli* B23 with nalidixic acid prior to transformation. Nalidixic acid is a member of the quinolone family of antibacterial agents. Quinolones inhibit DNA gyrase, a topoisomerase that negatively supercoils DNA (1). The minimum inhibitory concentration of nalidixic acid against *E. coli* is 0.3 µg/mL. Exposure of *E. coli* to sub minimum inhibitory concentrations results in formation of elongated cellular forms in which only DNA levels are markedly lowered, while there is no effect on protein and RNA synthesis (1). In this study, cell surface area was increased by sublethal exposure to nalidixic acid, and the efficiency of transformation relative to surface area was explored.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* K12 wild-type strain B23 was grown either on Luria-Bertani (LB) agar (5) at 37ºC for 16 to 20 hours or in LB broth (5) at 200 rpm at 37ºC for 16 to 20 hours for overnight cultures. To select for bacteria which contained the pUC19 vector (Fermentas) during the transformation experiments, ampicillin (Sigma-Aldrich) was added to the LB agar at 100 µg/mL.

Plasmids. The pUC19 vector was obtained from Fermentas (#SD0061). pUC19 (Genbank accession number L09137) is a high copy number vector which confers ampicillin resistance via the *bla* gene which encodes a beta-lactamase.

Minimum inhibitory concentration assay. 200 µL of an overnight *E. coli* B23 culture was diluted into 5 ml LB broth in 6 test tubes. Nalidixic acid (Negram, Winthrop-Breon) was added to each test tube to give a final concentration of 0, 0.25, 0.5, 1, 2, or 10 µg/ml. These cultures were incubated at 200 rmp at 37ºC and at 30, 60, 90, and 120 minutes after the start of incubation, samples were taken from each culture and viewed at 400X magnification with a phase contrast microscope (Zeiss Axiostar) to assess the shape and length of cells. The MIC was defined to be the minimum concentration of nalidixic acid at which the bacteria were observed to be approximately twice the length of the untreated bacteria. Images were taken using a Kodak DC290 Zoom digital camera.

Preparation of competent cells. 0.5 ml of an overnight *E. coli* K12 B23 culture was diluted into 49.5 mL LB broth, and this

culture was incubated at 37ºC at 200 rpm for 1.5 hour such that the OD_{600} was 0.15. The culture was then divided into two, the control cells and the nalidixic acid-treated cells. Nalidixic acid (Negram, Winthrop-Breon) was added to the second culture to a final concentration of 0.5 µg/mL and both cultures were then incubated at 37ºC at 200 rpm for 2 hours to give a final OD_{600} between 0.4 and 0.5. These cells were harvested by centrifuging both cultures at 3500 xg at 4ºC for 10 minutes and decanting the supernatant. Cells were placed on ice for all the intervening steps. Each cell pellet was resuspended in 5 ml ice cold 50 mM $CaCl_2$ and incubated on ice for 10 minutes. The mixtures were centrifuged at 3500 xg at 4ºC for 10 minutes and the supernatant was decanted. Each cell pellet was then resuspended in 0.75 ml ice cold 50 mM $CaCl_2$ + 15% glycerol solution. The cell mixtures were transferred to microfuge tubes in 50 µL aliquots via the use of an ethanol-dry ice bath to immediately freeze the cells. The competent cells were stored at -20ºC.

Viability tests. For pre-heat shock viability test, a 50 µL tube of competent cells was thawed on ice for 30 minutes, and 1 ml of LB broth was added to the tube. This culture was then diluted and plated in duplicate on LB agar which was incubated at 37ºC for 16 to 20 hours for a pre-heat shock viability test by counting colonies on each plate. For post-heat shock viability test, a 50 µL tube of competent cells was thawed on ice for 30 minutes, heat shocked at 42ºC for 2 minutes, and then incubated on ice for 2 minutes. 1 ml of LB broth was added to the tube and this culture was then diluted and plated in duplicate on LB agar which was incubated at 37ºC for 16 to 20 hours. The colonies on each plate were counted.

Transformation. A 50 µL tube of competent cells (nalidixic acid treated and untreated) was thawed on ice and 0, 1, or 3 µg of pUC19 vector was added to the tube. This mixture of cells and vector was incubated on ice for 30 minutes. The cells were then heat shocked at 42ºC for 45 seconds and then immediately incubated on ice for 2 minutes. 1 ml of LB broth was added to the cells, and the culture was incubated at 37ºC at 200 rpm for 1 hour. This culture was then plated in duplicate on LB agar which contained 100 µg/mL ampicillin to select for transformants. The LB agar was incubated at 37ºC for 16 to 20 hours and the colonies on each plate were enumerated. A no transformation control was conducted in which a 50 µL tube of untreated competent cells was used and which differs from the previously described protocol in that no 45 second heat shock step was completed.

RESULTS

Minimum inhibitory concentration of nalidixic acid. To determine a sublethal concentration of nalidixic acid that produces elongated cells, *E. coli* B23 were grown in the presence of a range of nalidixic acid concentrations. After 2 hours, cells grown in 0.25 to 1.0 µg/mL nalidixic acid were observed to be elongated (Fig. 1B), compared to cells grown in the absence of nalidixic acid (Fig. 1A). Cells grown in concentrations of 2.0 to 10.0 µg/mL nalidixic acid appeared small and irregular compared to the controls (Fig. 1C). Based on these results, a 0.25 µg/mL dose of nalidixic acid was selected to test the effects of antibiotic-induced cell elongation on transformation efficiency.

Competent cell viability. The viability of nalidixic acid-treated and untreated calcium chloride-competent B23 was determined before and after heat shock (FIG. 2). After calcium chloride treatment, the untreated *E. coli* contained 8.8 times as many viable cells as the nalidixic acid-treated stock. The mean concentration of viable untreated cells increased by 34% following the two minute 42˚C heat shock, from 2.62 x 10^{10} cfu/mL to 3.50 x 10^{10} cfu/mL. The mean concentration of viable treated cells was decreased by 89% following heat shock, from 29.7 x 10^{8} cfu/mL to 3.18 x 10^{8} cfu/mL.

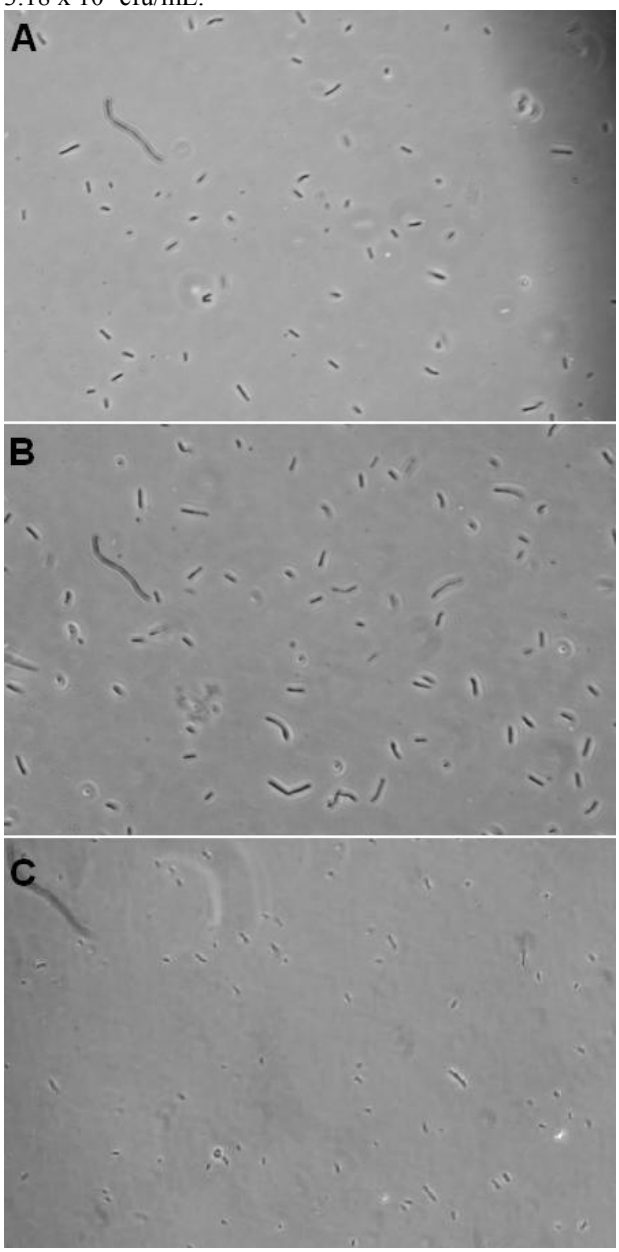

FIG. 1. Minimum inhibitory concentration assay of nalidixic acid on *E. coli* B23. Bacteria were grown at 37˚C and 200 RPM for 2 hours in the presence of (A) no nalidixic acid, (B) 0.25 µg/mL nalidixic acid, or (C) 10.0 µg/mL nalidixic acid.

Transformation efficiency. Treated and untreated competent cells were transformed with pUC19 plasmid encoding ampicillin resistance and

transformants were enumerated on LB plates containing ampicillin. Transformation frequencies of the treated and untreated samples are reported in Table I. The untreated sample produced 88 times as many transformants as the nalidixic acid-treated cells, and 9.5 times as many transformants per viable colony forming unit as the treated cells.

FIG. 2. Viability of competent *E. coli* B23 treated or untreated with nalidixic acid as measured by plate count assays. Viability determined before and after 2 minute 42˚C heat shock. Bars indicate 95% confidence intervals.

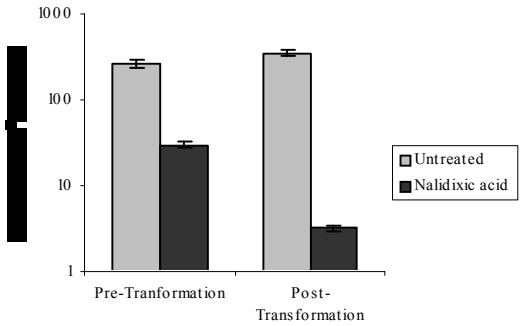

Table I. Transformation efficiency of Nalidixic acid-treated and untreated *E. coli* B23 with pUC19, as calculated by plate count assays on LB agar containing ampicillin.

Treatment	Transformants (cfu/μg)	Tranformants per viable cell ($x10^{-10}$ cfu/μg)
Untreated	140	53
Nalidixic acid	2*	6*

*All nalidixic acid values are estimates based on low colony counts showing a transformation frequency of less than 10 cfu/mL at a plasmid concentration of 3 μg/mL.

DISCUSSION

Contrary to our hypothesis, the nalidixic acid-induced elongation of the *E. coli* B23 cells lowered the transformation efficiency. The low number of transformed colonies observed and the consequent low transformation efficiency of the nalidixic acid-treated (Nal-treated) cells can be attributed to the low viability of the Nal-treated cells once they are subjected to heat shock for transformation. Our results indicated that approximately greater than 100% of the untreated cells and 10% of the Nal-treated cells survived the heat shock transformation method, thereby indicating that Nal-treated cells are more vulnerable to heat shock compared to untreated cells. This was also evident in temperature shift experiments performed by Zhao et al. on *E.coli* strain K12SH-28, which were treated with nalidixic acid (7). The *E.coli* K12SH-28 was subject to growth at 28°C and aliquots were taken and treated with nalidixic acid and the growth temperature was shifted to 42°C. The nalidixic acid treatment and temperature shift decreased the viability of the *E.coli* K12SH-28 (7). However, the low transformation efficiency for the transformed Nal-treated cells cannot be ascribed to the level of nalidixic acid chosen to treat the *E.coli* B23 cells. The level of nalidixic acid treatment of 0.25 μg/ml for 90 minutes had no affect on the viability of the *E.coli* B23 cells which can be observed in the viability test done prior to heat shock where the CFU counts of viable cells after treatment is similar to the CFU counts of untreated cells. This is consistent with the data that show that a treatment level of 5-20 μg/ml is needed in order to achieve the bactericidal effects of nalidixic acid (1). Therefore, a plausible reason for the low CFU counts of transformed colonies for the Nal-treated cells and the consequent low transformation efficiency is that heat shock has a considerable bactericidal effect on Nal-treated cells. This increased bactericidal effect could be due to the heat-shock/$CaCl_2$ treatment, which alters the cell membrane to allow for the passage of DNA. The treatment could have had a greater destabilizing effect on Nal-treated cells, as their membranes were twice the size of the untreated. This destabilizing effect on the membrane could have translated into the increased sensitivity of the Nal-treated cells.

Another contributing factor for the low CFU counts of transformed Nal-treated cells can be the interaction of nalidixic acid with LPS. As previously mentioned, the zones of adhesion that are rich in negatively charged LPS bind to divalent cations to facilitate the uptake of DNA (6). However, nalidixic acid has a carboxyl functional group that is anionic at neutral pH and has shown significant adsorption to positively charged alumina (3). Therefore, the treatment with the nalidixic acid could have bound Ca^{2+} as the divalent cation in $CaCl_2$ treatment and inhibited the binding of the plasmid DNA to the LPS via the divalent cation thereby decreasing the transformation efficiency. However, this would have had to occur with leftover trace elements of nalidixic acid as the $CaCl_2$ solution used for preparing competent cells was added after the cells were treated with nalidixic acid. Therefore, the hindrance of DNA binding to the cell and consequent low transformation efficiency could possibly be due to the interaction of nalidixic acid with LPS.

Overall, the transformation efficiency of nalidixic acid treated cells was lower than that of the untreated cells; however, the role of elongation of the cells by

means of nalidixic acid on transformation is still inconclusive. The role of nalidixic acid is inconclusive because the heat shock duration used for the actual transformation of both the control and Nal-treated cells is different from that used for the viability test. The consequence of the different durations used is that there is no means of quantitatively comparing the ratio of the yield of transformants relative to the number of viable cells post-heat shock for the treatment and the control. This would have allowed for the normalization of the number of transformants (CFUs) with respect to the effect of heat shock on viability and would have given more insight into the efficacy of nalidixic acid.

Therefore, the effectiveness of the nalidixic acid treatment still remains questionable. However, it is evident that nalidixic acid lowers the viability of *E.coli* B23 after the cells undergo heat-shock and $CaCl_2$ treatment.

FUTURE EXPERIMENTS

The proposed explanation for the increased sensitivity of Nal-treated cells to heat-shock and $CaCl_2$ treatment can be tested by using UV treatment to elongate the cells. UV treatment and heat-shock/ $CaCl_2$ treatment can be used to elongate and transform the cells. The use of UV treatment would allow for the separation of the effects of nalidixic acid from the effects of the increase in surface area. If a decrease in viability is observed after the heat-shock/ $CaCl_2$ treatment, then this would provide more evidence to our explanation that elongated cells are prone to killing from the treatment because their membranes are more greatly destabilized due to the increase in their size. Other alterations that can be done to make this experiment more effective would be to keep the duration of heat shock for the viability test and the transformation constant. This would allow for a more thorough analysis of the efficacy of nalidixic acid treatment by taking into consideration the effect of heat shock on the nalidixic acid-treated cells. In addition, the amount of time that was used for the heat shock in the viability test demonstrated to be too great for the nalidixic acid-treated cells, therefore the duration of heatshock should be reduced down from the 2 minutes used originally.

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