ANTIBACTERIAL PROPERTIES OF ZnO NANOPARTICLES AT LOW CONCENTRATION AGAINST ENTEROHAEMORRHAGIC ESCHERICHIA COLI (EHEC)

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A Dios quien en su inmensa misericordia, siempre ha puesto luz a mi camino

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ABSTRACT

TITLE: Antibacterial properties of ZnO nanoparticles at low concentration against *Enterohaemorrhagic Escherichia coli (EHEC)*[◆]

AUTHOR: Mauricio José Báez Atuesta[◆◆]

KEYWORDS: EHEC, ZnO NPs, Bacteriostatic behavior, ROS, LEE genes, Biofilm.

The ineffective treatment of some infection diseases with conventional antibiotics has caused that a significant number of researchers point out their effort to seek new materials with antibacterial properties, which may constitute a new generation of antibiotics. Hence, the aim of this thesis is to determine the antibacterial behavior of ZnO nanoparticles at low concentration and to evaluate the possibility of using them to treat the infection by Enterohaemorrhagic *Escherichia coli (*EHEC). The obtained results allow us to establish that at low concentration ZnO nanoparticles act as a bacteriostatic agent against EHEC. Their behavior is characterized by a delay in the bacterial growth without killing bacteria. Parallel experiments showed that at tested concentration, the generation of Reactive Oxygen Species (ROS) was not evident. The experiments also showed that the photo-activation of ZnO NP by the exposition to UV light did not have any effect onto the antibacterial behavior. Additionally, it was evaluated the effect of ZnO NPs onto the pathogenesis of EHEC infection. In-vitro adherence assay and the quantitative real-time PCR showed that at 250 μM, the treatment with ZnO NPs decreases the EHEC cells attached to HCT-8 cells and reduces the amount of RNA transcribed from LEE genes. It is important to highlight that the bacterial growth was not inhibited at tested concentration.

Based on the fact that EHEC infectious dose is extremely low (<100 cells), the bacteriostatic behavior of ZnO NPs at low concentration is not enough to counteract the expression of infection by a decrease of bacterial cells number. However, the findings about the ZnO effect on the pathogenesis of infection open the possibility to carry out further experiments, which consolidate ZnO NPs as a powerful inhibitor of infection by EHEC.

◆ Under-graduated thesis work carried out in Texas A&M University, College Station, Texas, USA.

◆◆Physical-Chemical Engineering Faculty, Chemical Engineering Department. Dir. Dr. Arul Jayaraman, Dr. Hung-Jue Sue. Reader. Dr Álvaro Ramírez García.

RESUMEN

TITULO: Antibacterial properties of ZnO nanoparticles at low concentration against *Enterohaemorrhagic Escherichia coli (EHEC)*[◆].

AUTOR: Mauricio José Báez Atuesta

PALABRAS CLAVES: EHEC, Nanoparticulas Oxido de Zinc, comportamiento bacterio-estático, genes LEE, Bio-películas

La inefectividad en el tratamiento de algunas enfermedades infecciosas con antibióticos convencionales ha impulsado a muchos investigadores hacia la búsqueda de nuevos compuestos con propiedades anti-bacteriales que constituyan una nueva generación de antibióticos. Consecuentes con la problemática actual, el principal objetivo de este trabajo de grado es determinar el comportamiento anti-bacterial de nanoparticulas de Oxido de Zinc (ZnO NP) a bajas concentraciones y a su vez evaluar la posibilidad de usarlas para combatir la infección producida por Enterohaemorragic *Escherichia coli* (EHEC). Los resultados obtenidos permitieron establecer que a baja concentración el oxido de Zinc actúa como un agente bacterio-estático contra la EHEC. Su comportamiento es caracterizado por inducir un retraso en el crecimiento bacterial sin causar inhibición de crecimiento o muerte celular. Experimentos adicionales mostraron que la generación de Especies Reactivas de Oxigeno (ROS) proveniente del ZnO no fue evidente a las concentraciones evaluadas, y que la foto-activación de las nanoparticulas de ZnO con rayos UV, no favoreció el desempeño antibacterial. Adicionalmente, fue evaluado el efecto de las nanoparticulas de oxido de Zinc sobre la patogénesis de la infección por EHEC. Ensayos de adherencia *in-vitro* y análisis de la expresión de ARN mostraron que a 250 µM, las nanoparticulas de oxido de Zinc disminuyen la adhesión de la EHEC a células de mamíferos y reducen la cantidad de ARN transcrito desde los genes responsable de la infección (genes LEE). Es importante resaltar que la concentración evaluada no inhibe el crecimiento bacterial.

Teniendo en cuenta que el número de células de EHEC necesario para desarrollar la infección es muy bajo, el efecto bacterio-estatico de las nanoparticulas de oxido de zinc a baja concentración no es suficiente para contrarrestar la infección mediante la disminución del número de células. Sin embargo, el comportamiento observado sobre la patogénesis abre la posibilidad de llevar a cabo futuras investigaciones que permitan consolidar las nanoparticulas de ZnO como un potente inhibidor de la infección por EHEC.

◆ Proyecto de grado desarrollado en Texas A&M University, College Station, Texas, USA

◆◆ Facultad de Fisicoquímicas, Escuela de Ingeniería Química. Dir. Dr Arul Jayaraman, Dr Hung-Jue Sue. Lector Dr. Álvaro Ramírez García.

INTRODUCTION

In recent years, the nanotechnology has been the response to problems in the biomedical and pharmaceutical areas. Since it has made possible the preparation of particles with specific requirements in terms of size; shape and physical, chemical and biological properties. Due to re-emergence of infectious diseases and the appearance of antibiotic-resistant strains within both, Gram-positive and Gram-negative microorganism (9), the Nanotechnology efforts have pointed out the development of nanomaterials with antibacterial properties. Those nanomaterials can be broadly classified into two types, organic and inorganic. Organic antibacterial materials are often less stable particularly at high temperature and/or pressures, compared to inorganic antibacterial agents (30). Thus, the uses of inorganic antibacterial agents such as metal and metal oxides have became the new generation of bactericidal materials (21, 24, 33).

Among these metal oxides, ZnO has attracted a special attention and it has been the protagonist in a lot of previous research. For instance, *Roselli et al (29)* established that ZnO protects enterocytes cells from *Enterotoxigenic E. coli* (ETEC) infection by inhibiting the adhesion and internalization of bacteria. Recently, *Crane et al (8)* found that Zinc acts as inhibitor agent of RNA expression from LEE genes in *Enteropathogenic e. coli* (EPEC), decreasing the expression of its virulence factor. In biomedical area, *Lee et al (19)* concluded that ZnO nanorods are potentially useful as an adhesion-resistant biomaterial, capable of reducing viability in anchorage-dependent cells. It is important to note that in previous research any effect was attributed to antibacterial behavior of ZnO.

Conversely, other authors have documented the behavior of ZnO as antibacterial agent, *Sawai et al (30)* showed the viability of using ZnO as a biocidal agent, and determined the dependence of antibacterial activity on agent concentration, same findings have been established in other researches (14, 16). The influence of size

particles have caused controversy between authors; while *Yamamoto (41)* found that the antibacterial activity of ZnO increased with decreasing particle size*, Adams et al (1)* reported that the size of nanoparticles do not affect its toxicity. Additionally, other authors have studied the effect on antibacterial behavior when using small molecules and macromolecules to control the size and shape of nanoparticles (5). Interestingly, some results have shown selective toxicity of ZnO nanoparticles to prokaryotic and eukaryotic system, even it has been proved preferential killing of cancer cells using ZnO (13, 28). Overall, the cited authors have demonstrated the viability of ZnO as antibacterial agent relating its behavior with properties such as concentration, stability and dispersion in the bacterial culture medium, among others.

Concerning mechanism, it is thought that the nanoparticles can either interact directly with the microbial cells, e.g. interrupting transmembrane electron transfer, disrupting/penetrating the cell envelope, oxidizing cell components, or produce secondary products (e.g. reactive oxygen species (ROS) or dissolved heavy metal ions) that cause damage. In our case, the death of bacteria in the presence of ZnO nanoparticles appears to be caused by membrane disruption, possibly arising from the formation of ROS. Close contact between ZnO nanoparticles and bacterial cell and internalization of ZnO NP into the cell cytoplasm have been demonstrated (5, 14).

On other hand, Enterohaemorrhagic *Escherichia coli* (EHEC) is a human pathogen that colonizes the large intestine and causes attaching and effacing (AE) lesions on intestinal epithelial cells. These bacteria are highly infectious and ingestion of only 100–200 organisms is sufficient to trigger debilitating diarrheal disease. Although most EHEC infections resolve spontaneously after 5–10 days of abdominal cramping and bloody diarrhea, approximately 2–7% of cases progress to the potentially–fatal haemolytic uremic syndrome due, in part, to the production of

cytotoxic Shiga toxins which are capable of promoting kidney failure. At present, there is not an effective medical mechanism to treat the EHEC infection.

The aim of this work was to determine the behavior of ZnO NPs as antibacterial agent at low concentration, and to evaluate the possibility of using them against E*nterohaemorrhagic E. coli (EHEC)* infection.

1. THEORY

1.1 ZINC OXIDE NANOPARTICLES FEATURES.

ZnO is an important and attractive semiconducting material. It has drawn enormous research attention due to its distinguished properties optics, photonics and electronics. Its wide bandgap energy (3.4 eV) at room temperature is ideal for short-wavelength optoelectronic applications. Compared with other wide bandgap semiconductors, ZnO has several advantages. As the size decreases to the regime in between the bulk and isolates molecules, the properties of semiconductor nanocrystals may have mechanical, optical, electric and thermal properties quite different from the bulk, known as the quantum size effect. Another factor accounting by the crystal size is the large surface to volume ratio, which also results in some properties being dominated by the surface rather than the crystalline core.

Usually the synthesis of ZnO nanoparticles can be carried out in different types of alcoholic solutions, i.e., methanol, ethanol, propanol, or higher alcohols. The unique features of methanol over other alcohols in the synthesizing ZnO nanoparticles involve its high dielectric constant and low ligand affinity. The dielectric constant of a solvent primarily determines its polarity. Usually, the higher the dielectric constant is, the higher the polarity of the solvent. On one hand, electrolytic salts have high solubility in high-polarity solvents, but are generally hard to be dissolved in non-polar solvents, as known as “like dissolves like”. For instance, $Zn(Ac)_2{\cdot}2H_2O$, the most commonly used zinc salt for synthesizing ZnO

nanoparticles, has a higher solubility in methanol than in other alcohols. On the other hand, methanol, with a higher polarity, gives rise to a faster formation of ZnO nanoparticles than in methanol under non-basic condition. During the growth of ZnO nanoparticles, the alcohols not only provide the medium for the reactions, but also act as ligands to help the control the morphology of ZnO, i.e., particles or rods. Under hydrothermal conditions, ZnO has a higher tendency to form rods in ethanol than in methanol, which makes methanol a good solvent for growing spherical ZnO nanocrystals. Moreover, methanol is a good solvent for dissolving quite a few monomers and polymers. Therefore direct handling of ZnO nanoparticles in methanol offer more versatility for the preparation of polymer/ZnO nanocomposites.

1.2 ENTEROHAEMORRHAGIC ESCHERICHIA COLI (EHEC)

Escherichia coli (E. coli) is a bacterium that is commonly found in the gut of humans and warm-blooded animals. Most strains of E. coli are harmless. Some strains however, such as enterohaemorrhagic E. coli (EHEC), can cause severe foodborne disease. It is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk. Its significance as a public health problem was recognized in 1982, following an outbreak in the United States of America. EHEC produces toxins, known as verotoxins or Shiga-like toxins because of their similarity to the toxins produced by Shigella dysenteriae. EHEC can grow in temperatures ranging from 7°C to 50°C, with an optimum temperature of 37°C. Some EHEC can grow in acidic foods, down to a pH of 4.4, and in foods with a minimum water activity (Aw) of 0.95. It is destroyed by thorough cooking of foods until all parts reach a temperature of 70°C or higher. E. coli O157:H7 is the most important EHEC serotype in relation to public health; however, other serotypes have frequently been involved in sporadic cases and outbreaks

1.2.1 INFECTION MODE

Enterohaemorrhagic *Escherichia coli* O157:H7 (EHEC) colonizes the human colon, resulting in the development of hemorrhagic colitis and hemolytic-uremic syndrome that may be fatal. Upon colonization of the colon, EHEC forms attaching and effacing (AE) lesions on the epithelial cells and produces Shiga toxin. These lesions efface the microvilli and rearrange the cytoskeleton to form their signature pedestal-like structure, which cups each bacterium. The genes responsible for the formation of AE lesions are located on a pathogenicity island named the locus of enterocyte effacement (LEE). The LEE comprises 41 genes organized in five major operons, LEE1, LEE2, LEE3, tir (LEE5) and LEE4. Included in the LEE are *sep* and *esc* genes, encoding a type III secretion system (TIIISS); the *eae* gene, encoding intimin; *tir*, encoding the translocated intimin receptor; the *espABD* genes, which encode proteins that are secreted by the type III secretion system; and *ler* (LEE-encoded regulator), which encodes an H-NS-like protein that activates the expression of the LEE genes. The EHEC interaction with the epithelial of the gastrointestinal tract can be briefly summarized as follow: EHEC moves through the gastrointestinal mucus using rotating flagella. By sensing environmental signals, EHEC “primes” itself for attachment by producing hair like fimbriae on its surface. As it was explained above the EHEC genome contains integrated DNA islands that encode virulence factors, one of them is known as LEE, which is made up of 5 polycistronic operons. Initially, each operon is blocked by H-NS, a DNA binding protein, but the H-NS repression is lifted by an activator protein, called as Ler. It displaces H-NS, freeing up the operon promoter sites allowing that RNA polymerase can now transcribe the DNA into mRNA.

The structural proteins of the T3SS from LEE1-3 are now translated and assembled at the bacterial inner membrane to produce the basal apparatus of the T3SS, while the LEE4 and LEE5 are processed separately. Using its fimbriae, EHEC can attach to the microvilli of gut epithelial cells. This contact triggers further

gene expression event inside the bacterium such as the formation of proteins from LEE4 mRNA and their translation at the basal apparatus for their secretion.

The resultant translocon proteins EspA, EspD and EspB from LEE4, are exported with the aid of chaperones. Firstly, EspA polymerises to form a long hollow filament from the outer bacterial membrane to the membrane host cell, through this filament EspD and EspB pass to host cell. These proteins form a pore in the membrane of the host epithelial cell. Now, the bacterium is able to translocate its own proteins from inside the bacteria through into the host cell.

Meanwhile, Intimin is inserted into the bacterial outer membrane, Tir is translocated into the host cell, phosphorylated and inserts into the host cell membrane. In these conditions EHEC can now form a tight association with the host cell by the interaction of the bacterial protein Intimin and its translocated receptor, Tir. This attachment causes the recruitment of other proteins to the site leading to the re-arrangement of the host cell cytoskeleton. Consequently, the microvilli are effaced and the bacterium is partially embedded in the host cell membrane. This is known as an Attaching and Effacing lesion and it promotes the persistence of the bacteria in the gut. The last step of this process is characterized by the release of toxins, which can induce bloody diarrhea and can lead to life threatening kidney damage.

1.2.2 DISEASES BY EHEC

Symptoms of the diseases caused by EHEC include abdominal cramps and diarrhea that may in some cases progress to bloody diarrhea (haemorrhagic colitis). Fever and vomiting may also occur. The incubation period can range from three to eight days, with a median of three to four days. Most patients recover within 10 days, but in a small proportion of patients (particularly young children and the elderly), the infection may lead to a life-threatening disease, such as haemolytic uraemic syndrome (HUS). HUS is characterized by acute renal failure, haemolytic anaemia and thrombocytopenia. It is estimated that up to 10% of patients with EHEC infection may develop HUS, with a case-fatality rate ranging from 3% to 5%.

Overall, HUS is the most common cause of acute renal failure in young children. It can cause neurological complications (such as seizure, stroke and coma) in 25% of HUS patients and chronic renal sequelae, usually mild, in around 50% of survivors.

2. EXPERIMENTAL SECTION

2.1 PREPARATION OF POLYVINYLPYRROLIDONE -CAPPED ZNO (ZNO-PVP) NANOPARTICLES.

PVP-capped ZnO Nanoparticles were prepared by hydrolyzing Zn $(O_2CCH_3)_2.H_2O$ in a basic methanol solution with the presence of PVP. The synthesis and purification were performed according to a previously described protocol by Sun at el (38) except that 1.4506 g of PVP (MW 8000, Sigma-Aldrich) were also dissolved in methanol containing KOH before the reaction. Briefly, 200 ml of methanol containing 0.9775 g of KOH (99.99%, Aldrich, Milwaukee, WI) and 1.4506 g of PVP (MW 8000, Sigma-Aldrich) were first heated at 60 °C for 30 min to obtain a homogeneous solution. Powder of 0.008 mol $Zn(O_2CCH_3)_2.H_2O$ (99%, Fluka, Castle Hill, Sweden) was then directly added into a basic methanol solution to make the final $[Zn^{2+}]$ of 0.04 M. This starting solution was allowed to react at 60 °C for 2 hrs with stirring and refluxing. After reaction, the ZnO solution was concentrated 10 times *via* rotary evaporation under vacuum at 40 °C. Subsequently, hexane and isopropanol were added into the concentrated ZnO solution with a volume ratio of hexane: isopropanol: methanol of 5:1:1. The ZnO Nanoparticles precipitated immediately after adding hexane and isopropanol. This mixture was kept at 0°C overnight until the ZnO Nanoparticles were fully precipitated and settled at the bottom of the container. The above-mentioned operation was repeated twice to purify the ZnO Nanoparticles and the remaining solvents were fully removed *via* rotary evaporation under vacuum to avoid contamination. Twenty ml of deionized and distilled water were added into the dried PVP-ZnO Nanoparticles, and after ultrasonification, a gel-like sample containing ZnO-PVP Nanoparticles was obtained. This gel-like sample was used to prepare a

stock solution of PVP capped-ZnO Nanoparticles at 1 mg/ml, in water-PVP. The mass ratio in solution of PVP: ZnO was 300:1. The sample solutions used during this research were made by dilution of stock solution.

The ZnO Nanoparticles powder X-ray diffraction (XRD) patterns were performed by a Bruker D8 Advance Powder X-ray Diffractometer with Cu-K∞ incident radiation (λ = 1.5418 Å). The patterns were recorded at 0.04 ° per step and step time of 6 s.

2.2 BACTERIAL STRAINS, MAMMALIAN CELL LINES AND MATERIALS

E. coli O157:H7 (CDC EDL933) and *E. coli* BW25113 were obtained from ATCC (Manassas, VA). Plasmids pCM18 (Clontech, California) was used to constitutively express the green fluorescent protein (GFP). Luria-Bertani medium (LB) was used for overnight cultures of *E. coli* O157:H7 (ATCC, Manassas, VA). The human colon cancer cell line, HCT-8 (ATCC, Mansasses, VA), derived from enterocytes at the junction of large and small bowel, was maintained in RPMI 1640 medium supplemented with 10 % horse serum.

2.3 DETERMINATION OF THE ANTIBACTERIAL ACTIVITY OF ZNO NANOPARTICLES

In this study *E. coli* O157:H7 was used since it is a zoonotic enteric pathogen of worldwide importance. Antibacterial test was performed by measuring the bacterial growth curve in a Luria-Bertani media in the presence of ZnO different concentrations. *E. coli* O157:H7 obtained from a glycerol stock was inoculated into 15 ml of LB media and grown aerobically at 37 ° C overnight with shaking (200 rpm). The saturated culture was diluted into three Erlenmeyer flasks with fresh LB medium (OD_{600}=0.05) containing different concentrations of ZnO nanoparticles (ZnO NPs) (250, 750, 1500 µM). Cultures grew at 37 ° C with vigorous shaking at 200 rpm. The growth curves were determined by measuring the time evolution of the optical density (OD). The measurements were done at 600 nm with spectrophotometer (Beckman DU 640) at a frequency of once an hour. Previously,

bacterial growth curves were performed by determination of remaining cells numbers after the treatment with ZnO nanoparticles. The experimental scheme was similar to the described above except that the ZnO NPs concentrations were 75, 150, 300 and 600 μM and the measurement were done at different time points (4, 8, 16, 24, 48 h). Remaining number of cells were determining by plating samples.

To determine the general behavior of ZnO as antibacterial agent at low concentration the same antibacterial test was carried out using specific conditions. In this research, it was tested the effect of some parameters such bacterial medium and UV light photo-activation on antibacterial behavior of ZnO Nanoparticles. To compare the efficacy of ZnO nanoparticles in rich medium (LB) and minimal medium, EHEC obtained from glycerol stock was inoculated into a tube with 5 ml of LB media and grown overnight at 37^{o}C with shaking, then the overnight culture was re-inoculated in M9 minimal media OD_{600}=0.05, ZnO Nanoparticles were added to a final concentration of 75 and 250 μM. Samples were collected every 8h for 24h and the number of cells were determined by plating.

The behavior of ZnO Nanoparticles in M9 minimal media was tested at short time exposure too. The EHEC overnight culture was diluted into fresh LB medium (OD_{600}=0.05) and grew aerobically at 37°C for three hours with shaking (200 rpm), cell pellets were obtained and washed with phosphate buffer solution (sterile PBS, pH 7.2) twice, then the cell pellets were re-inoculated into two tubes containing 5 ml of M9 minimal and ZnO Nanoparticles at 250 and 1500 μM respectively, the number of cells was determine by plating after 1 hour.

2.3.1 UV LIGHT PHOTO-ACTIVATION OF ZnO NANOPARTICLES SOLUTION

The influence of UV light photo-activation of ZnO nanoparticles was determined as follow, PVP capped-ZnO Nanoparticles solution in LB medium was prepared into three Erlenmeyer flasks; then, the solutions were exposed to UV light during different times (10, 20, 30 minutes), and growth analysis was performed with a ZnO

concentration of 250 μM. EHEC was inoculated not only in control but also in photo-activated ZnO containing flasks. The cell number was determined by plating samples after 4 hours.

2.3.2 NITROBLUE TETRAZOLIUM REDUCTION ASSAY (REACTIVE OXYGEN SPECIES ROS)

In order to further establish the role of oxidative stress in the antibacterial activity of ZnO Nanoparticles, the production of Reactive Oxygen species in samples treated with ZnO Nanoparticles was determined by *Nitroblue tetrazolium reduction* (NBT assay), it was adapted from that described by Barnes et al (3). A volume of 100 μl of bacterial suspension in LB media (OD_{600} 1.0) was incubated with ZnO Nanoparticles solution at different concentrations (250, 750 1500 μM) and 100 mg/ml nitroblue tetrazolium (NBT) solution at final concentration of 0.2 % for 30 min at 37 °C. Then, 5 μl of 2 M HCl was added and the tubes were centrifuged at 1500×*g per* 10min. Absorbance of supernatants was measured at 575 nm (extracellular ROS). Finally, 50 μl of LB were added to separated pellets and absorbance was determined at 575 nm (intracellular ROS).

2.3.3 VARIATION OF MASS RATIO OF PVP: ZNO NANOPARTICLES IN SOLUTION

The ZnO Nanoparticles synthesis and the preparation of stock solution were performed as the described above, except that the mass ratio PVP: ZnO in solution was modified by changing the final concentration of ZnO-PVP Nanoparticles. The normal ratio was increased 18, 12 and 6 times respectively. The PVP-ZnO nanoparticles solutions were used to carry out a new antibacterial test. It was done determining the remaining number of cells after a six-hour treatment with PVP-ZnO NP solution at 250 μM. Cells number was determined by plating cells.

Subsequently, other antibacterial test was carried out using the PVP-ZnO NP solution with the highest PVP-ZnO ratio, but in this case the bacterial growth curve was obtained by measuring the time evolution of the optical density (OD). Since the

concentrations of the stock solutions were lower than the original stock solution used in former experiments, it was used a higher volume of ZnO-PVP solution. According to that, it was used two different control samples: without any solution and with a PVP solution to analyze a possible effect of PVP. The preparation of bacterial suspension was done by following the procedure described in the first paragraph of ***Determination of the antibacterial activity of ZnO Nanoparticles*** in this paper.

2.4 IN VITRO ADHESION ASSAYS.

Adhesion of EHEC to HCT-8 cells was adapted from that described by Bansal et al (2) who used HeLa cells. HCT-8 cells (ATCC, Manassas,VA) were routinely cultured and propagated in serum RPMI media according to standard protocols (ATCC). Low-passage-number HCT cells were cultured in standard 24-well tissue culture plates and grown at 37°C in a 5% CO2 humidified environment until ~ 80% confluence. HCT-8 cell monolayers were washed twice with sterile PBS to remove unattached cells, and the growth medium was replaced with antibiotic-free RPMI with 10% heat-inactivated HS. Approximately 200 cells of freshly grown *E. coli* O157:H7 (turbidity of ~ 0.8) and 250 μM ZnO were added to wells and incubated for 3 hours at 37^{o}C in a 5% CO_2 environment. Loosely attached *E. coli* O157:H7 cells were removed by washing the wells thrice with sterile PBS, and the HCT-8 cells were lysed in the wells using 0.1% Triton X-100 dissolved in PBS. The cell suspension in each well was vigorously vortexed, and serial dilutions of the bacteria were plated on LB agar plates. Colonies were counted after 24 h incubation at 37^{o}C.

2.5 QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION.

Quantitative RT-PCR was performed using a Bio-Rad iCycler (Bio-Rad, CA) real-time RT PCR unit (18). Approximately 50 ng of total RNA from control or ZnO-treated *E. coli* O157:H7 was used for reverse transcription. Gene sequences were downloaded from the *A Systematic Annotation Package for Community Analysis of*

Genomes, (ASAP) website (https://asap.ahabs.wisc.edu/asap/home.php?formSubmitReturn=1) and gene-specific primers were used for *eae, espA, escC, escV* and *rpoA* (housekeeping control). Threshold cycle numbers were calculated using the MyiQ software (Bio-Rad), and PCR products were verified using agarose electrophoresis. qRT-PCR experiments were performed in triplicate.

2.6 BIOFILM FORMATION IN SIMPLE MICROFLUIDIC DEVICES.

A single colony of E. coli BW25113/pCM18 capable of producing GFP (Green Fluorescent Protein) was inoculated LB media and grown overnight, the saturated culture was re-inoculated in M9 minimal media by 4 hours (OD_{600} 1). The bacterial growth in both media was performed at 37 °C with shaking (200 rpm) and was necessary to use 300 mg/ml erythromycin to preserve the plasmid. Then bacterial cells were seeded into the two channels of the microfluidic device at 37 °C in a 5% CO2 humidified environment for 2 hours. The microfluidic device was washed with sterile PBS prior to cell seeding.

Sterile syringes were filled with 50 % M9 minimal media and bacterial solution (OD_{600} 1.0) and flowed through each channel of microfluidic device in a 37 °C in a 5% CO2 humidified environment at 0.03 ml/h using a multi-syringe pump. After 9 hours the established Biofilm was imaged using Zeiss Axiovert 200M inverted fluorescence microscope (Carl Zeiss Inc. Thornwood, NY) with a FITC filter setting. The two syringes with M9 minimal media were changed by new filled syringes with ZnO Nanoparticles solution in 50 % TB media at 250 and 1500 µM respectively and the bacterial flow was closed. The established Biofilm was exposed to ZnO solution for four hours, and then the images were taken using the conditions mentioned previously. Simple micro-channel devices were used for all preliminary device experiments.

3. RESULTS

3.1 CHARACTERIZATION OF ZnO NANOPARTICLES

The efficiency in the antibacterial behavior of some metal nanoparticles depend on their physicochemical properties, thus the appropriate changes in some of them, such as size and surface characteristics, could modulate their antibacterial performance. PVP-capped ZnO Nanoparticles obtained through the precipitation-redispersion procedure exhibit optimal size particle and a high crystalline structure. The use of PVP during synthesis offers an important advantage because it is used as a template not only to control the size particles, but also to favor the distribution of ZnO Nanoparticles in solution. **Figure 1** shows the XRD pattern of ZnO- PVP Nanoparticles. It exhibits a good Wurtzite structure and the diffraction peaks corresponding to hexagonal-type ZnO appeared around 31.7°, 34.6° and 36.9° in the sample. Due to extremely small particle size, the spectrum of our ZnO is broader than other ZnO nanoparticles and the characteristic peaks tend to overlap. Using the Debye-Scherrer formula $t = \frac{0.89\lambda}{\beta \cos\theta}$; (t stands for the average diameter of particles; λ, the X-ray wavelength; β, full width at half maximum of the diffraction peak; and θ, the diffraction angle) the average size particles were around 5 nm.

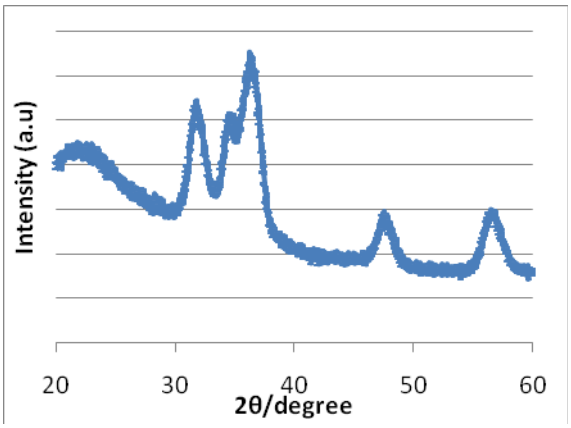


Figure 1. XRD patterns of ZnO Nanoparticles

3.2 ANTIBACTERIAL BEHAVIOR OF ZnO NANOPARTICLES

The majority of methods used to evaluate the antibacterial behavior of a material, can be summarized as follow: by counting the remaining bacterial cells after the treatment with the material, and by measuring the time evolution of the optical density (OD) in the treated sample. As they were described in ***Determination of the antibacterial activity of ZnO Nanoparticles***, it was used both methods. **Figure 2** shows the efficiency of ZnO nanoparticles measured and obtained as normalized ratio of growth by the first method described above. Evidently, the effect of ZnO nanoparticles depends on concentration. The lowest ratio of growth was obtained at 600 μM and it became higher while the concentration became less. Other factor which deserves special attention is the time of the effect. Although at 600 μM the ratio of growth was the lowest, the duration of the effect was even shorter than the sample treated with ZnO nanoparticles solution at 75 μM. The obtained curves described clearly a bacteriostatic behavior characterized by the antibacterial agent, which does not inhibit the bacterial growth but delays it.

The results obtained by measuring the culture turbidity (**Fig. 3**) confirm the previous observations. The dependence of concentration was evident. For instance at 1500 μM of ZnO, the lag phase was extended up to 3 hours, and the slope of curve in the log phase was significantly less. Although in this case it was used a range of concentration broader than the previous experiment, the behavior at tested concentrations was similar to it.

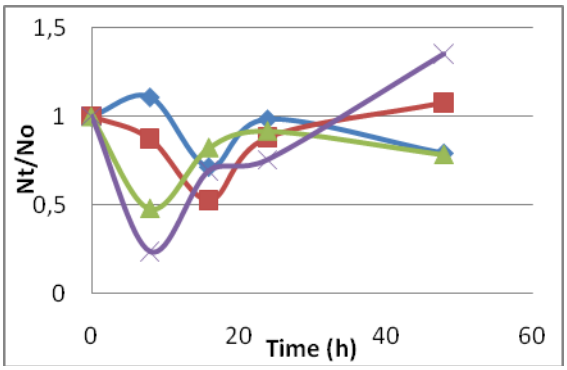


Figure 2. Antimicrobial activity of ZnO NPs at different concentrations as a function of time in LB media. The antimicrobial activity was measured as a normalized growth ratio. Nt is equivalent to Number of EHEC cells in treated samples. No is equivalent to Number of EHEC cells in control sample (75 µM; 150 µM; 300 µM; 600 µM)

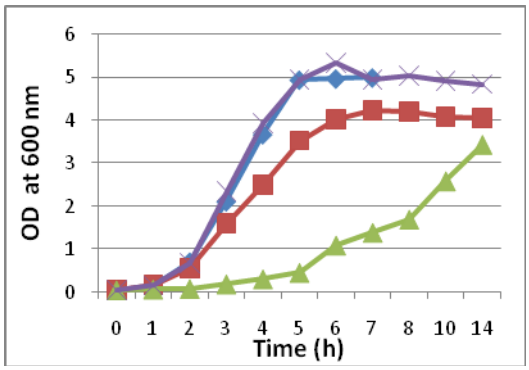


Figure 3. EHEC growth curve at different ZnO NPs concentrations. (0 µM; 250 µM; 750 µM; 1500 µM)

Taking into account that this is an exploratory research, it was tested different conditions trying to find the best performance of ZnO Nanoparticles at low concentration. In our antibacterial tests using LB media, the main problem was that, the number of bacterial cells remaining after ZnO treatment was sufficient to make them grow back. Accordingly, in this media the bacterial growth rate could hide the antibacterial effect. Hence, the used media was changed from LB (rich media) to M9 minimal media, to determine if the EHEC growth rate is greater than the anti-bacterial rate. It was formulated two experiments in M9 minimal media changing the time exposure.

Figure 4 shows the behavior of ZnO Nanoparticles in M9 minimal media at long time exposure. The ability of ZnO was measured as normalized ratio of growth and two concentrations of ZnO Nanoparticles were tested (75 and 250 µM). The data clearly showed that the antibacterial activity of ZnO is independent from concentration. The lowest ratio of growth at both concentrations was about 0.4. Although, there were differences after 8 hours where at 250 µM the bacterial growth was slower than at 75 µM. The antibacterial behavior was similar to LB media. Once more, the action mode of ZnO was bacteriostatic and it was not

enough because the number of remaining cells after the treatment still remained high.

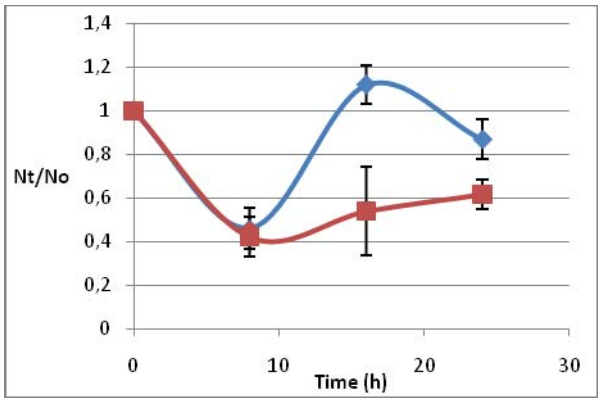
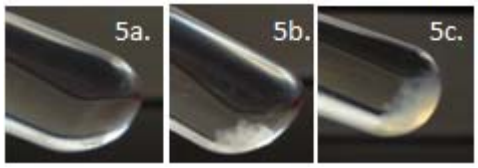


Figure 4. Antibacterial activity of ZnO NPs at different concentrations as a function of time in M9 minimal media. The antimicrobial activity was measured as a normalized growth ratio. Nt is equivalent to Number of EHEC cells in treated samples. No is equivalent to Number of EHEC cells in control sample (75 µM; 250 µM).

The previous results obtained in M9 minimal media, force us to investigate the reasons why the antibacterial activity is independent of concentration, and to improve the conditions which allow us to isolate the effect of EHEC growth rate. Consequently the second antibacterial test in M9 minimal media was carried out following the procedure outlined in ***Determination of the antibacterial activity of ZnO Nanoparticles***. The results showed that the behavior in M9 minimal media was not caused by antibacterial properties of ZnO but rather other additional factors (**Figure 5**). Although after the ZnO NPs treatment, apparently dead bacterial cells were deposited in the bottom of the tubes, it were not found significant differences in the cells number of treated samples and the control, thus the formed precipitate were not dead cells but it could be a secondary product of undesirable reaction.



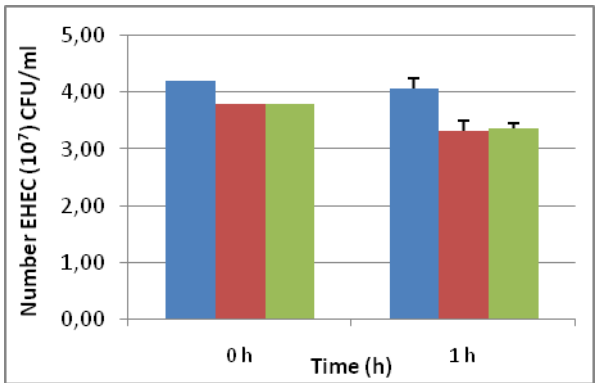


Figure 5. Antibacterial activity of ZnO at different concentrations in M9 minimal media (short time exposure). After ZnO NP treatment for 1 hour was evident the formation of a precipitate in the treated samples at 250 µM (5b) and 1500 µM (5c), unlike control (5a). There was no evidence of this precipitate was death bacteria cell. Since the remaining cells number after ZnO treatment for 1hour was constant.

3.2.1 EFFECT OF PHOTO-ACTIVATION OF ZnO NP WITH UV LIGHT

In the use of nanoparticles as antibacterial agents, one of principal challenge is to understand the mechanism as they attack the bacterial cells. ZnO is a semiconductor and can be excited by UV light. Within aerobic environment it could be "activated" and release electrons which could react with the available oxygen and form superoxide and others Reactive Oxygen Species (ROS). These species are involved in mechanism used by different nanoparticles against bacterial cells. Although different hypothesis have been suggested about this mechanism, the most accepted is to disrupt membrane activities through lipid peroxidation by ROS, such as superoxide (O_2^-), and hydroxyl radicals (^-OH)(27).

Formation of ROS, in absence of photons, for nanoparticles that present antibacterial activity has not been totally studied. Furthermore, before evaluating the generation of ROS in the samples treated with low concentration of ZnO Nanoparticles, it was estimated the possibility that the ZnO could photo-activate by exposure to UV light and increase its antibacterial effect. The results showed that the photo-activation of ZnO Nanoparticles solution did not have significant effect on its antibacterial ability (**Fig 6).** Even at the tested concentration, the cell number in the sample exposed at UV light for the longest time, after the treatment of ZnO, was slightly higher than the non-photo activated sample.

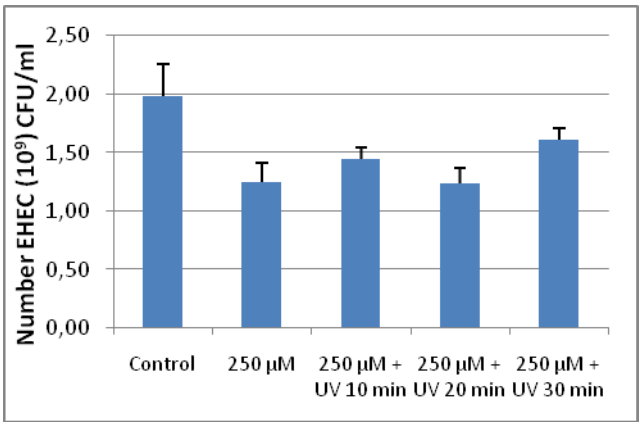


Figure 6. Influence of photo-activation with UV light on the antibacterial activity of ZnO NPs.

3.2.2 DETERMINATION OF THE INFLUENCE OF ROS

Based on the previous results, it is not easy to establish a relationship between photo-activation and the generation of ROS, which allows us to predict the effect of ZnO Nanoparticles on ROS production. Hence, it was formulated a specific assay using NBT solution to determine if at low concentration, the generation of ROS is responsible for ZnO Nanoparticles behavior as antibacterial agent. The results showed that the generation of ROS from samples untreated and treated with ZnO Nanoparticles were similar (**Fig 7**), which suggests that ROS production was not involved in the delay of bacterial growth. Since ROS are unstable molecules, their measurement is sensitive to conditions in environment, thus the results obtained are dependent on experimental conditions

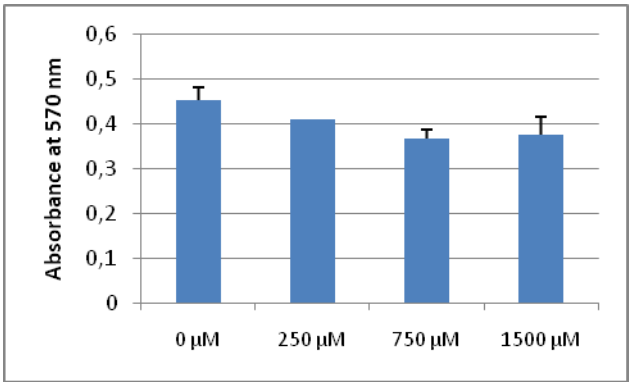


Figure 7. Determination of generated ROS from samples treated with different concentration of ZnO NPs

3.2.3 EFFECT THE VARIATION OF PVP: ZnO MASS RATIO IN SOLUTION

PVP was used as template to control the shape and size of ZnO Nanoparticles during synthesis reaction to molar ratio of PVP: ZnO of 1.5:1 and as dispersant and stabilizer of ZnO Nanoparticles in aqueous solution at mass ratio of 300:1. Previous research (5, 16, 41) have shown that nanoparticles with small size possess high antibacterial activity, and that the use of macromolecules do not affect the general antibacterial behavior. The extremely small size of our ZnO Nanoparticles suggests that the amount of PVP used during the synthesis is optimum. However, changes in the dispersion level of nanoparticles in solution could enhance the antibacterial activity and it synergistically acts with small size. In our case the dispersion level in solution depends on the mass ratio of PVP: ZnO. Consequently, it was varied this ratio and evaluated the effect on antibacterial action (**Fig. 8**). The results clearly suggest that the antibacterial activity is proportional at PVP: ZnO ratio in solution. Growth of bacterial cells resumed rapidly with a decrease in the ratio of PVP: ZnO. Consequently, the highest ratio of PVP: ZnO (sample 1) showed the best effect against EHEC.

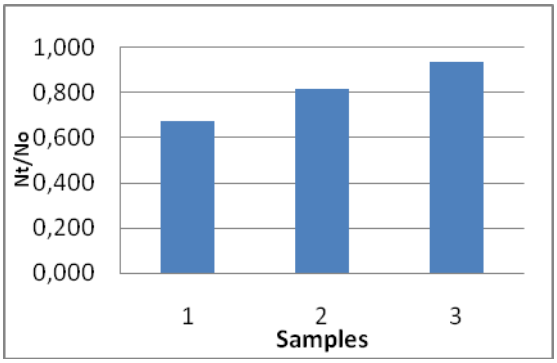


Figure 8. Effect of the increase in solution mass ratio PVP: ZnO on Antibacterial effect of ZnO NPs. The antibacterial activity was measured as a normalized growth ratio. Nt is equivalent to Number of EHEC cells in treated samples. No is equivalent to Number of EHEC cells in control sample. Sample 1: PVP/ZnO=5400; Sample 2: PVP/ZnO=3600; Sample3 PVP/ZnO=1200. The tested concentration was 250 µM.

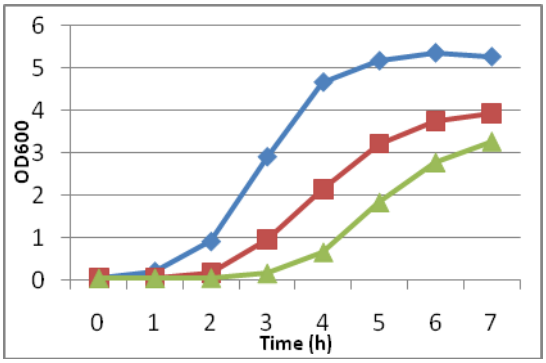


Figure 9. EHEC growth curve at 250 µM with high PVP/ZnO ratio using ZnO-PVP NP solution with PVP/ZnO ratio: 5400; control 1 EHEC solution; Control 2 EHEC solution+ PVP solution

The increase of solution ratio of PVP: ZnO did not improve the antibacterial effect of ZnO-PVP NP. Since, the normalized growth ratio in the most affected sample by

ZnO effect (sample 1) was similar to the normalizated growth ratio after treatment using a ZnO NPs solution with normal PVP: ZnO ratio. Despite of that, the appearance of sample 1 was not turbid up to five hours which suggested that the bacterial growth was so slow. Therefore, this hypothesis was validated by determination of bacterial growth curves using the same ZnO NPs solution of sample 1.

The **figure 9** shows the bacterial growth curves of treated sample and both controls. Evidently, the bacterial growth in the sample treated with ZnO NPs solution with high PVP/ZnO ratio was slower than the controls. There were not only changes in the lag phase but the exponentially phase was affected as well. It can be seen that the lag phase was extended up to two hours regarding the control and the differences between the slopes of each curve during exponential phase, letting us suppose that there were changes on growth rates. Our suggestions about possible effects of PVP were correct because it had an effect on the growth curve, although the effect of ZnO nanoparticles was evident, and it was more significant than others.

3.3 EFFECT OF ZnO NP ON THE EHEC ADHERENCE TO MAMMALIAN CELLS.

Infection diseases due to Enterohemorrhagic *Escherichia coli* (EHEC) are characterized by diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. The adherence of EHEC on epithelial cells is believed to be the first step for developing these diseases. There are some reports about adhesion mechanisms of EHEC on epithelial cells (25, 39). Thus, it was formulated an experiment to investigate if ZnO nanoparticles at a concentration of 250 μM can inhibit EHEC attachment to mammalian cells. **Figure 10** shows the treatment with 250 μM ZnO reduced EHEC adhesion to eukaryotic cells. These results suggest that ZnO-treated EHEC are less infective.

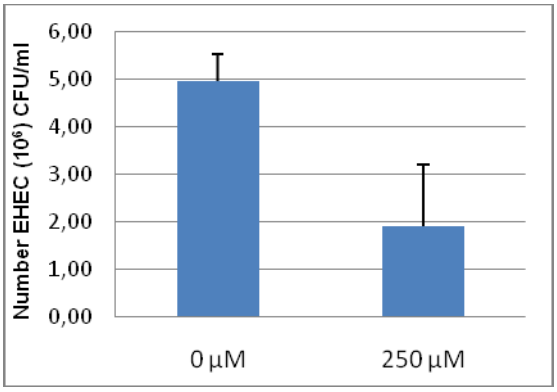


Figure 10. Reduction of Enterohemorrhagic *E .coli* (EHEC) adhesion to HCT-8 cells by Zinc Oxide.

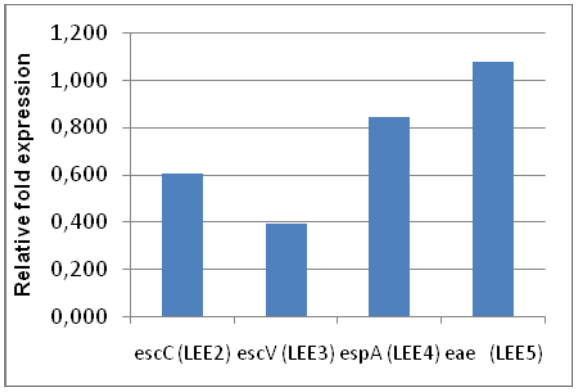


Figure 11. Effect of ZnO NPs on the abundance of RNA transcribed from genes in the LEE.

3.4 EFECT OF ZnO ON THE RNA TRANSCRIPTION FROM LEE GENES

The next step was to elucidate if the drop on the EHEC ability to adhere to mammalian cells by ZnO action was due to physical interference or it was due to changes on gene expression of genes responsible. The physical interference thought to be by ZnO-hindering the contact between bacterial and mammalian cells. Concerning gene expression, previous references have shown that the genes responsible for the expression of EHEC infection are located on a pathogenicity island named the Locus of Enterocyte Effacement (LEE) (17). It was chosen four LEE genes to investigate the molecular basis of effect of ZnO on adherence and others virulence factors. Real-time RT-PCR analyses provided interesting insights into how ZnO NPs affect the RNA transcription of LEE genes. **Figure 11** shows that ZnO nanoparticles decreased the RNA transcribed from *escC, escV* and *espA*. The ZnO's effect on *eae* was not evident.

3.5 EFFECT OF ZnO ON ESTABLISHED BIOFILM

In recent years, the microbial Biofilm has received much attention (4, 7, 23); in part owing to its presence in natural, industrial and clinical environments and its effect has been negative. Hence, for many researchers, to understand the morphological

characteristics have become a new challenge. For instance, a prime concern of medicine is the difficulty of eradicating Biofilm bacteria with antibiotic treatment. Taking into account the ideas above, it was formulated an experiment to evaluate the ZnO effect on established Biofilm using microfluidic devices. Although we have focused on ZnO nanoparticles effect on EHEC, it had to be used other *E.coli* strain to carry out this experiment due to the EHEC Biofilm is not common.

The formation of bacterial Biofilm on microchannels is a novel attempt to simulate the real behavior in dynamic conditions. With this kind of experiment we could be close to real behavior in human intestine. Previous to this experiment we tried to investigate whether ZnO nanoparticles could inhibit the formation of new Biofilm by coating microchannels' surface. Due to the fact that the coating was not correctly fixed on the surface, it was swept by solution flow and we could not get any results to infer the ZnO action. Regarding established Biofilm, the effect of ZnO nanoparticles is shown in **Figure 12**, the pictures allowed us see that the effect of ZnO nanoparticles on microbial Biofilm depends on concentration. Although, neither tested concentrations removed the Biofilm nor inhibited the formation of new Biofilm, the amount of Biofilm on the microchannel treated with the highest ZnO concentration was significantly lower than the Biofilm on the other microchannel. This is a promising result since it lets us formulate further experiments to evaluate other conditions and improve the obtained results.

a. b.

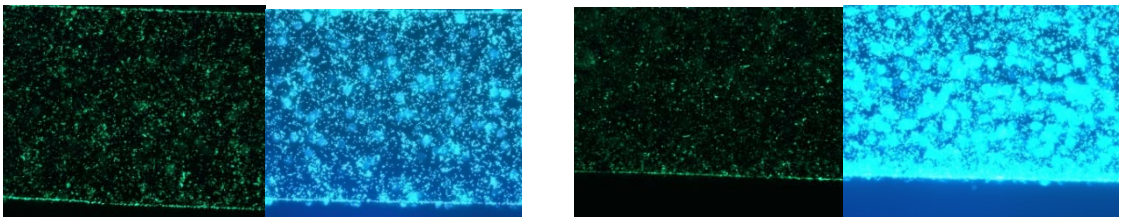


Figure 12. Effect of ZnO NPs on established *E. coli BW 25113* biofilm. (a) Pre and post treatment of ZnO at 1500 µM. (b) Pre and post treatment of ZnO at 250 µM.

4. DISCUSSION

During the past decade, the re-emergence of infection disease and the development of resistance of a variety of pathogen bacteria at treatment with antibiotics, has became a serious threat to public health and it has forced to a significant group of researchers to point out their effort to seek a new generation of antibiotics. Nanotechnology offers an important prospect since there is a possibility for the formulation of new materials with unique morphologic properties, which may lead to the development of new antibacterial agents. Recent advances in this field have been in the formulation of metallic oxide with biocide effect (1, 6, 24, 30, 37).

Among the metallic oxide nanoparticles, ZnO have shown a viable behavior as antibacterial agent and its properties have been studied extensively (5, 14, 16, 26, 28, 42). Researchers have concluded that ZnO nanoparticles are effective for inhibiting both, gram-positive and gram-negative bacteria. Even it has showed selective toxicity towards cancer cells, they have also shown that properties such as smaller size (under specific conditions(1)), high solution stability and high concentration, could enhance the antibacterial effect. On the topic of mechanism used by ZnO against bacteria cells, although it has not been understood completely, the membrane disruption by Reactive Oxygen Species (ROS) has been the most accepted. It is important to note that ZnO antibacterial activity has been reported only at very high concentrations which are not safe for human use. Besides, any of referenced authors have elucidated a behavior at low concentration.

Herein, we tried to accumulate evidence to suggest ZnO NPs as antibacterial agent against EHEC infection, which is responsible for grave intestine diseases. The main reason to evaluate the ZnO effect at low concentration was the EHEC condition as human pathogen bacteria, since it forced us to take into account the toxicity levels of ZnO in human cells. Previous reports (15, 20) showed that the cytotoxicity of ZnO depends on the cell type, dose and time. While *Jeng and*

Swanson demonstrated that nanoparticle-exposed Neuro-2A cells (especially ZnO) at doses >50 *μ*g/mL became abnormal cellular function and changes in cell morphology, *Lin et al* established on human lung epithelial cells that ZnO was cytotoxic at significant lower concentrations (> 15 *μ*g/mL). Obtained results of experiments carried out in Dr Mariah Hahn's Lab testing our ZnO nanoparticles (results not published yet), showed ZnO-citotoxicity at concentration > 20 *μ*g/mL. Consequently, the adherence assay and analysis of RNA expression were carried out at 250 *μM.*

Our results showed that at low concentration ZnO acts as bacteriostatic agent. These kinds of agents limit the growth of bacteria by interfering with some aspect of bacterial cellular metabolism. They can inhibit growth and reproduction of bacteria without killing them, thus the time of effect is an important factor since after it is over, the bacterial growth continues normally. **Figure 2** supports this definition, evidently the ZnO was only acting for a period of time, and then the bacterial growth resumed. Interestingly, the time effect at 300 and 600 *μ*M was lower than 75 and 150 *μ*M. It could be explained by considering that high concentrations can be known as cellular perturbations which are quickly controlled by defense system of bacteria cells to prevent adverse effects. Meanwhile, the effect of low concentrations is tolerated and controlled for the bacterial cell in its normal metabolism. This behavior suggests the presence of effective regulation-system to warrant the cell surviving. In *E. coli* different regulatory systems are known such as osmoregulatory system (40) and antioxidative system (11), which modulate the bacterial response under specific stimulus. These systems are important to elucidate a possible antibacterial effect since they act as a defense mechanism.

To discuss about possible mechanism used by ZnO NPs at low concentration, we have to make some observations to complement our main hypothesis. (I) Although, we did not prove the responsibility of ROS in the antibacterial behavior of ZnO

nanoparticles at low concentration, we do not rule out the possibility that they are related, since the indirect methods of measure could be inexact due to the high instability of these particles. Besides, the generation of ROS from ZnO and other photosensitive compounds has been proved previously (1, 21, 31). (II) Our ZnO NPs exhibit high stability and great level of dispersion in PVP/water matrix, but in the bacterial medium the redispersed ZnO NPs are unstable and they tend to agglomerate and even precipitate, hence the amount of suitable ZnO decrease considerably. *Brayner et al (5)* who used experimental conditions similar to ours, reported inhibition of bacterial growth by 85 % at ZnO concentration between 3.0 mM and 1.5 mM, while our reports at 1.5 mM showed a bacteriostatic behavior yet. (III) Due to the fact that the lifetime of ROS is about 10^{-6} to 10^{-5} seconds, the generation of ROS is not sufficient to warrant a biocidal effect. If ROS play a key role in the antibacterial effect, the nanoparticles that generate them must be close enough to bacterial cells to ensure the ROS diffusion into the cell. However, although the adsorption of QDs on surfaces of *E. coli* could possibly make ROS enter into a bacterial cell, their antibacterial effect still depends on the antioxidative response of *E. coli (22).*

By correlation of our results and previous conclusion, we proposed a mechanism to explain the ZnO NPs antibacterial effect at low concentration. We think that ZnO NPs could surround bacterial cells, and accumulate onto their cell membranes, triggering changes in the cell osmotic conditions and increasing the viscosity of the bacterial culture medium, which slow down the transport processes of oxygen and other nutrients between bacteria cells and the aqueous environment, resulting in a delay of the bacterial growth. However, this behavior is transitory and its duration depends on the velocity of the response mechanism to counteract the negative perturbation. We do not reject the possibility that adsorbed ZnO NPs onto the bacterial membrane cell could mediate the formation of ROS, but at low concentration the oxidative effect is not a threat to cellular viability and it is easily controlled. We infer that the accumulation of nanoparticles close enough to

bacterial cell constitute a viable cause of osmotic stress. Our hypothesis agrees with *Stecchini et al (35, 36),* who established that changes in the osmotic conditions and the mechanical constraints such as viscosity and diffusivity by presence of specific particles in the bacterial culture medium can restrict the bacterial growth and limit the cellular response to the biological effect of osmotic stress. Besides, the results shown in **figure 9** corroborate our hypothesis. The increase of PVP/ZnO ratio was reflected in a significant delay in the bacterial growth by extending the lag phase and decreasing the bacterial growth rate. It is so important to highlight that PVP does not have any antibacterial effect, as it was proved by *Zhang et al (42).* In our case, the observed effect was due to increase the viscosity of medium owing to the presence of high volume of PVP solution. This result suggests that further research should be to point out the investigation of co-materials, which work in synergy with ZnO and to dwindle the dependence of concentration. As it is seen in **Figure 9,** the bacterial growth curve at 250 *μ*M and high PVP/ZnO ratio was almost similar to the bacterial growth curve at 1500 *μ*M.

On the other hand, the effect of ZnO nanoparticles on RNA transcribed from LEE genes is an important result, since it makes viable the use of ZnO NPs against EHEC by indirect way, without decreasing the number of bacterial cells. As it is known, the LEE1 operon encodes a transcription activator of the other LEE operons that is called the LEE-encode regulator (Ler). It is essential for the expression of multiple LEE-located genes in both EPEC and EHEC, including those encoding the type III secretion pathway, the secreted Esp proteins, Tir, and intimin (10, 34). The reduction of the abundance of the RNAs encoded by escC *(LEE2), escV (LEE3)* and *espA (LEE4)* as result of treatment with ZnO NPs, suggest the previous inhibition of LEE1 expression and consequently of *ler* expression. Taking into account the *LEE1*, *LEE2*, and *LEE3* operons encode components of the type III secretion system (*esc* and *sep* genes) and the *LEE4* operon encodes the secreted proteins EspA,-D, and –B, all of which are responsible of main EHEC virulence factors. We may infer that these results are

related to the decrease in the adherence of EHEC in eukaryotic cells. This finding is of great importance in preventing the EHEC infection. ZnO NPs did not have effects on the expression of eae (LEE5). We expected that this gen may have a similar behavior to other genes, since it is known that *ler* regulates the LEE5 expression too. *Haack et al* (12) established that, although *LEE5* is regulated by *ler,* the *ler* regulation of LEE5 is more complex and requires specific environment conditions inside the human gut. This may explain why the inhibitory effect of ZnO, on the expression of *eae,* was not evident.

Overall, our results suggest that the treatment with ZnO NPs reduce the infective condition of EHEC cells by affecting the LEE genes expression. Similar results were found by *Crane et al* (8) on EPEC bacteria, who additionally proposed a mechanism of action of Zinc, which could be mimicked in EHEC

Finally, the effect of ZnO NPs on the Biofilm formation could not be to clearly elucidate, our experiment was at first attempt to know a general response but it did not give enough bases to suggest a possible mechanism. However, the results constitute a promissory onset to carry out further experiments.

5. CONCLUSIONS

I. We concluded that ZnO NPs act as bacteriostatic agent against Entherohaemorrhagic *E. coli* (EHEC) at low concentration. Its behavior is characterized by the delay of bacterial growth without triggering bacterial cell death.

II. It was inferred that the bacteriostatic behavior is caused by accumulation of ZnO NPs onto bacterial cell membrane and its surroundings, which slow down the process of oxygen and vital nutrients transportation between bacterial cell and the environment.

III. It was proved that the photo-activation of ZnO nanoparticles by exposition at UV light does not enhance their antibacterial properties.

IV. The bacteriostatic action is not enough to combat the EHEC infection, since the number of cells remaining after ZnO NPs treatment is sufficient to make it grow back even if ZnO had an efficiency of 90 %. This suggests that decreasing EHEC infections by a decrease number of cells is not effective.

V. On the other hand, it was demonstrated that the treatment with ZnO NPs decreases the amount of RNA encoded by *LEE* genes. Consequently, it reduces the capability of EHEC cells to evolve the infection. It constitutes a promissory result, since it opens the possibility to carry out further experiments to consolidate ZnO nanoparticles as powerful inhibitor of infection by EHEC. We recommend the use of DNA microarray instead of Real time RT-PCR. Since the DNA microarray allows to evaluate the behavior of a higher number of genes.

VI. Based on our hypothesis about the mechanism used by ZnO NPs at low concentration, we think that additional experiments may contribute to accumulate more evidence. We recommend using analysis of RNA or DNA expression to determine accurately, if EHEC cells undergo any kind of stress during the exposition to ZnO Nanoparticles.

VII. Additionally, we think that the use of Scanning Electronic Microscopy (SEM) or Transmission Electronic Microscopy (TEM) may contribute to probe the interaction between the bacterial cells and ZnO Nanoparticles.

6. REFERENCES

1. **Adams, L. K., D. Y. Lyon, and P. J. J. Alvarez.** 2006. Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions. water research **40:**3527-3532.
2. **Bansal, T., D. Englert, J. Lee, M. Hegde, T. K. Wood, and A. Jayaraman.** 2007. Differential Effects of Epinephrine, Norepinephrine, and Indole on Escherichia coli O157:H7 Chemotaxis, Colonization, and Gene Expression. Infect. Immun. **75:**4597-4607.
3. **Barnes, A. I., I. L. Herrero, and I. Albesa.** 2005. New aspect of the synergistic antibacterial action of ampicillin and gentamicin. International Journal of Antimicrobial Agents **26:**146-151.
4. **Bayles, K. W.** 2007. The biological role of death and lysis in biofilm development. Nat Rev Micro **5:**721-726.
5. **Brayner, R., R. Ferrari-Iliou, N. Brivois, S. Djediat, M. F. Benedetti, and F. Fievet.** 2006. Toxicological impact studies based on Escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Lett **6:**866-870.
6. **Chang, Q., L. Yan, M. Chen, H. He, and J. Qu.** 2007. Bactericidal Mechanism of Ag/Al2O3 against Escherichia coli. Langmuir **23:**11197-11199.
7. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. Science **284:**1318-1322.
8. **Crane, J. K., T. M. Naeher, I. Shulgina, C. Zhu, and E. C. Boedeker.** 2007. Effect of zinc in enteropathogenic Escherichia coli infection. Infect Immun **75:**5974-5984.
9. **Desselberger, U.** 2000. Emerging and re-emerging infectious diseases. J. Infect **40:**3-15.

10. **Elliott, S. J., V. Sperandio, J. A. Giron, S. Shin, J. L. Mellies, L. Wainwright, Hutcheson, S. W., T. K. McDaniel, and J. B. Kaper.** 2000. The Locus of Enterocyte Effacement (LEE)- Encoded Regulator Controls Expression of Both LEE- and Non-LEE-Encoded Virulence Factors in Enteropathogenic and Enterohemorrhagic *Escherichia coli*. Infect. Immun. **68:**6115-6126.
11. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol. Mol. Biol. Rev. **55:**561-585.
12. **Haack, K. R., C. L. Robinson, K. J. Miller, J. W. Fowlkes, and J. L. Mellies.** 2003. Interaction of Ler at the LEE5 (tir) Operon of Enteropathogenic Escherichia coli. Infect. Immun. **71:**384-392.
13. **Hanley, C., J. Layne, A. Punnoose, K. M. Reddy, I. Coombs, A. Coombs, K. Feris, and D. G. Wingett.** 2008. Preferential killing of cancer cells and activated human T cells using ZnO nanoparticles. Nanotechnology **19:**295103.
14. **Huang, Z., X. Zheng, D. Yan, G. Yin, X. Liao, Y. Kang, Y. Yao, D. Huang, and B. Hao.** 2008. Toxicological effect of ZnO nanoparticles based on bacteria. Langmuir **24:**4140-4144.
15. **Jeng, A. H., and J. Swanson.** 2006. Toxicity of Metal Oxide Nanoparticles in Mammalian Cells. Journal of Environmental Science and Health Part A **41:**2699-2711.
16. **Jones, N., B. Ray, K. T. Ranjit, and A. C. Manna.** 2008. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. FEMS Microbiology Letters **279:**71-76.
17. **Kendall, M. M., D. A. Rasko, and V. Sperandio.** 2007. Global Effects of the Cell-to-Cell Signaling Molecules Autoinducer-2, Autoinducer-3, and Epinephrine in a *luxS* Mutant of Enterohemorrahagic *Escherichia coli.* Infect. Immun. **75:**4875-4884.

18. **Lee, J., T. Bansal, A. Jayaraman, W. E. Bentley, and T. K. Wood.** 2007. Enterohemorrhagic Escherichia coli Biofilms Are Inhibited by 7-Hydroxyindole and Stimulated by Isatin, p. 4100-4109. vol. 73.
19. **Lee, J., B. S. Kang, B. Hicks, T. F. Chancellor Jr, B. H. Chu, H.-T. Wang, B. G. Keselowsky, F. Ren, and T. P. Lele.** 2008. The control of cell adhesion and viability by zinc oxide nanorods. Biomaterials **29:**3743-3749.
20. **Lin, W., Y. Xu, C.-C. Huang, Y. Ma, K. B. Shannon, D.-R. Chen, and Y.-W. Huang.** 2008. Tocixity of nano- and micro-sized ZnO particles in human lung epithelial cells. J. Nanopart Res.
21. **Lovric, J., S. J. Cho, F. M. Winnik, and D. Maysinger.** 2005. Unmodified Cadmium Telluride Quantum Dots Induce Reactive Oxygen Species Formation Leading to Multiple Organelle Damage and Cell Death. Chemistry & Biology **12:**1227-1234.
22. **Lu, Z., C. M. Li, H. Bao, Y. Qiao, Y. Toh, and X. Yang.** 2008. Mechanism of Antimicrobial Activity of CdTe Quantum Dots. Langmuir **24:**5445-5452.
23. **Mah, T.-F. C., and G. A. O'Toole.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology **9:**34-39.
24. **Makhluf, S., R. Dror, Y. Nitzan, Y. Abramovich, R. Jelinek, and A. Gedanken.** 2005. Microwave-Assisted Synthesis of Nanocrystalline MgO and Its Use as a Bacteriocide. Advanced Functional Materials **15:**1708-1715.
25. **Nagano, K., K. Taguchi, T. Hara, S.-i. Yokoyama, K. Kawada, and H. Mori.** 2003. Adhesion and Colonization of Enterohemorrhagic Escherichia coli O157: H7 in Cecum of Mice. MICROBIOLOGY and IMMUNOLOGY **47:**125-132.
26. **Nair, S., A. Sasidharan, V. Divya Rani, D. Menon, S. Nair, K. Manzoor, and S. Raina.** 2008. Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells. Journal of Materials Science: Materials in Medicine**:**3548-3552.

27. **Neal, A. L.** 2008. What can be inferred from bacterium-nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles? Ecotoxicology **17:**362-371.

28. **Reddy, K. M., K. Feris, J. Bell, D. G. Wingett, C. Hanley, and A. Punnoose.** 2007. Selective tocixicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems. Appl. Phys Lett **90:**21390-21391_21390-21393.

29. **Roselli, M., A. Finamore, I. Garaguso, M. S. Britti, and E. Mengheri.** 2003. Zinc oxide protects cultured enterocytes from the damage induced by Escherichia coli. J Nutr **133:**4077-4082.

30. **Sawai, J.** 2003. Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. Journal of Microbiological Methods **54:**177-182.

31. **Sawai, J., S. Shoji, H. Igarashi, A. Hashimoto, T. Kokugan, M. Shimizu, and K. Hiromitsu.** 1998. Hydrogen Peroxide as an Antibacterial Factor in Zinc Oxide Powder Slurry. Journal of Fermentation and Bioengineering **86:**521-522.

32. **Sia, S. K., and G. M. Whitesides.** 2003. Microfluidic devices fabricated in Poly(dimethylsiloxane) for biological studies. ELECTROPHORESIS **24:**3563-3576.

33. **Sondi, I., and B. Salopek-Sondi.** 2004. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. Journal of Colloid and Interface Science **275:**177-182.

34. **Sperandio, V., J. L. Mellies, R. M. Delahay, G. Frankel, J. A. Crawford, W. Nguyen, and J. B. Kaper.** 2000. Activation of enteropathogenic *Escherichia coli* (EPEC) LEE2 and LEE3 operons by Ler. Molecular Microbiology **38:**781-793.

35. **Stecchini, M. L., M. Del Torre, I. Sarais, O. Saro, M. Messina, and E. Maltini.** 1998. Influence of Structural Properties and Kinetic Constraints on Bacillus cereus Growth. Appl. Environ. Microbiol. **64:**1075-1078.

36. **Stecchini, M. L., M. Del Torre, and E. Venir.** 2004. Growth of Listeria monocytogenes as influenced by viscosity and water activity. International Journal of Food Microbiology **96:**181-187.
37. **Stoimenov, P. K., R. L. Klinger, G. L. Marchin, and K. J. Klabunde.** 2002. Metal Oxide Nanoparticles as Bactericidal Agents. Langmuir **18:**6679-6686.
38. **Sun, D., M. Wong, L. Sun, Y. Li, N. Miyatake, and H.-J. Sue.** 2007. Purification and stabilization of colloidal ZnO nanoparticles in methanol. Journal of Sol-Gel Science and Technology **43:**237-243.
39. **Winsor, D. K., Jr., S. Ashkenazi, R. Chiovetti, and T. G. Cleary.** 1992. Adherence of enterohemorrhagic Escherichia coli strains to a human colonic epithelial cell line (T84). Infect. Immun. **60:**1613-1617.
40. **Wood, J. M., H. Dieter, and S. Helmut.** 2007. Bacterial Osmosensing Transporters, p. 77-107. Methods in Enzymology, vol. Volume 428. Academic Press.
41. **Yamamoto, O.** 2001. Influence of particle size on the antibacterial activity of zinc oxide. International Journal of Inorganic Materials **3:**643-646.
42. **Zhang, L., Y. Jiang, Y. Ding, M. Povey, and D. York.** 2007. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). Journal of Nanoparticle Research **9:**479-489.

ATTACHMENT 1 Characteristics of Bacterial growth [a]

Although widely varying in morphology, bacteria share one major characteristic: they divide by simple **binary fission**. This means that one cell grows to about double its original size and then splits into two genetically identical cells. Since DNA replication occurs before the cells divide, each new cell, called a daughter cell, gets a complete genome (a full set of genes). The two genetically identical daughter cells are called **clones.** All the progeny of a single original cell form a mass of cells on a solid surface as agar that is called a colony. If the original form was not a single cell, for instance, it was aof cocci, that entire chain of cells and all its progeny will form a single colony. So a **colony forming unit (CFU)** may include the progeny of a single cell, or it may include the progeny of several cells that were originally connected to each other.

The mathematics of bacterial growth is fairly simple, since each original cell divides to form two new cells, with the loss of the original parent. Since once cell become two and as each of those cells divides they become four and then eight, etc., the mathematical series describing growth is: 1, 2, 4, 8, 16, 32, 64 … This can be written as 2^0, 2^1, 2^2, 2^3 ...Therefore, this is a serie in base 2. It is an exponential series, since the number that increase in the series is the exponent. Thus, bacteria show exponential growth.

Exponential growth leads to rapidly increasing populations. For instance, a bacterium that divides every 30 min has a **generation time** of 30 min. every 30 minutes the population doubles. Although theoretically bacteria would increase over a hundred-trillion-fold, they do not grow to such a high population density due to their growth becomes limited as the population density increase.

Bacterial growth curve

Bacterial growth over time can be graphed as cell number versus time. This is called a **growth curve**. The cell number is plotted as the log of the cell number,

since it is an exponential function. Regardless of the generation time, in a growing culture the plot of the log of cell number versus time gives a characteristic curve. This curve typically has four distinct phases: lag phase, exponential (log) phase, stationary phase, and death phase.

In cells that have been freshly inoculated into a new growth medium, the **lag** phase is the first phase observed. It is characterized by no increase in cell number, however, the cells are actively metabolizing, in preparation for cell division. Depending on the growth medium, the lag phase may be short or very long. For instance, if a culture in a rich growth medium that supplies most of the cells' requirements is inoculated into a poor medium that requires the cells to make most of their own amino acids and vitamins, the lag phase will be very long. The cells must activate the metabolic pathways for amino acid and vitamins synthesis and must make enough of these nutrients to begin active growth. In contrast, cells that are simply diluted from one medium to a fresh tube of the same medium may show virtually no lag phase, since they need not change their metabolism.

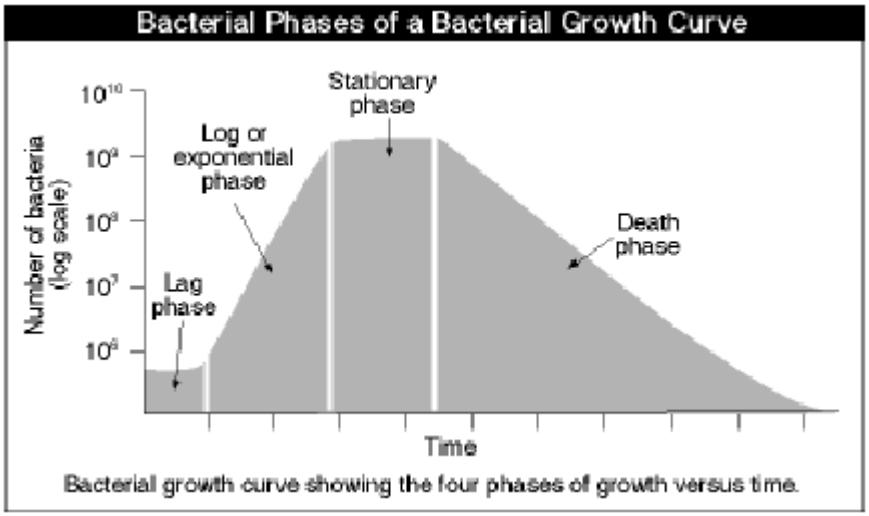


Figure 13. Bacterial Phase of a Bacterial Growth Curve

Once cells are actively the begin DNA replication and shortly after that the cells divide. This begins the second phase of growth called the **exponential or log phase** of growth. This is the period in which the cells grow most rapidly, doubling at

a fairly constant rate. The time it takes the culture to double is called the **generation time**. The generation time can be easely obtained from the exponential phase of a growth curve. The log of the cells number versus time will yield a straight line when the cells are in exponential growth. The generation time can be read directly from the graph using two points on the straight line that represent a two-fold increase in the cell number.

The generation time depends on several factors: the organism itself, the growth medium, and the temperature are all important factors in determining the generation time. Under constant conditions, the generation time for any organism is quite reproducible, but differs greatly among different bacteria. The fastest growing bacteria have generation time of 15-20 min under optimum growth conditions. Many bacteria, however, have generation time of hours or even days.

The third phase in the growth of bacteria is **stationary phase,** when metabolism slows and the cells cease rapid cell division. They may divide slowly for a time, but soon stop dividing completely. They are still alive and maintain a slow metabolic activity. The factors that cause cells to enter stationary phase are related to changes in the environment, typically caused by high cell density. Among the changes that slow growth are depletion of nutrients and accumulation of waste products. If cells in stationary phase are diluted into fresh medium they quickly resume exponential growth.

The final phase of the growth cycle is the **death phase.** In this phase the cells quickly lose the ability to divide even if they are placed in fresh medium. Like the phase of rapid growth, the death phase is also exponential; therefore, cells die quickly and within hours a culture may have no living cells. The death phase, and in fact all the phases. Can be slowed by lowering the temperature. Hence, **in order to maintain maximum cell viability it is best to grow bacterial cultures only to early stationary phase and then chill the culture.** Leaving a culture at the optimum temperature for growth for a long period of time simply accelerates the

death of the culture. Most dead bacterial cells look identical to live cells, so the normal appearance of a liquid culture or of colonies on a plate is no indication that the cells are alive.

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[a] Information and figure taken from Teresa Thiel. Science in the real world: Microbes in action 1999

ATTACHMENT 2 Quantitative Reverse-Transcription Polymerase Chain Reaction: Main features

Definition

Real time PCR or quantitative PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA (mRNA) in a sample. Using sequence specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. We use the term relative since this technique tends to be used to compare relative copy numbers between tissues, organisms, or different genes relative to a specific housekeeping gene. The quantification arises by measuring the amount of amplified product at each stage during the PCR cycle. DNA/RNA from genes with higher copy numbers will appear after fewer melting, annealment, and extension PCR cycles. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles.

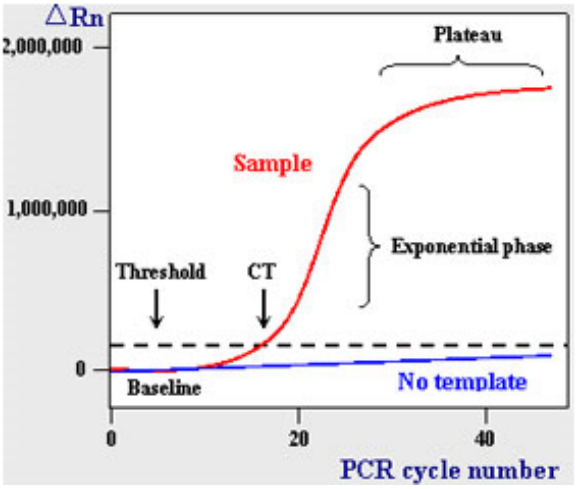

Figure 14. Amplification plot of mRNA

Quantification

The diagram above shows a typical reading from a single PCR cycle in a real time PCR machine. The vertical axis represents copy number (arbitrary units) and the horizontal axis shows the PCR cycle number (ie, how many

melting/annealment/extension cycles have occurred). The dotted threshold line is an arbitrary value, usually about 0.1 and is the "copy number" used to determine Ct. The lower a Ct value, the more copies are present in the specific sample. When plotted on a linear scale, as above, the curve has a sigmoidal course with an exponential phase and a plateau phase. The plateau phase is really determined by the amount of primer in the master mix rather than the nucelotide template. Usually the vertical scale is plotted in a logarithmic fashion, allowing the intersection of the plot with the threshold to be linear and more easily visualized. Theoretically, the amount of DNA doubles every cycle during the exponential phase, but this can be affected by the efficiency of the primers used. A negative control is always performed with no template to show a lack of intrinsic fluorescence. A positive control using a housekeeping gene that is relatively abundant in all cell types is also performed to allow for comparisons between samples. Typical housekeeping genes include 18S rRNA, GAPDH, and actin. When real time PCR is combined with reverse transcriptase PCR (RT-PCR), mRNA can be quantified for an assessment of relative gene expressions between tissues or genes. The amount of DNA/RNA is determined by comparing the results to a standard curve produced by serial dilutions of a known amount of DNA/RNA.

Quantification methods

Some sort of reporter method is required to be able to quantify amplified product after each PCR cycle. A popular method is the use of double-stranded DNA dyes. These dyes nonselectively bind to all double-stranded DNA, resulting in fluorescence. dsDNA dyes such as SYBR Green are nonselective such that they will bind to any dsDNA, including primer dimers. Another method of PCR product quantification is the use of a fluorescent reporter probe. This is more accurate since the probe is designed to be sequence specific and will only bind to the specific PCR product. The probe specificity allows for quantification even in the presence of non-specific DNA amplification. This allows for multiplexing (assaying several genes in the same reaction) by using probes with different coloured labels.

Typically, this method uses an RNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the other end. Breakdown of the probe by 5' to 3' exonuclease activity of taq polymerase removes the quencher and allows the PCR product to be detected. TaqMan probes are the most commonly used.

ATTACHMENT 3 Techniques for Bacterial Cell Culture: Media preparation and plating method.[a]

Basic information for the culture of bacteria is presented here, using *Escherichia coli* as a representative organism. The techniques and recipes should be readily transferrable.

The supplier of the particular bacterium being cultured will recommend an appropriate medium.

Escherichia coli is a rod-shaped bacterium with a circular chromosome about 3 million base pairs (bp) long. It can grow rapidly on *minimal medium* that contains a carbon compound such as glucose (which serves both as a carbon source and an energy source) and salts which supply nitrogen, phosphorus, and trace metals. *E. coli* grows more rapidly, however, on a *rich medium* that provides the cells with amino acids, nucleotide precursors, vitamins, and other metabolites that the cell would otherwise have to synthesize.

When *E. coli* is grown in liquid culture, a small number of cells are first *inoculated* into a container of sterile medium. After a period of time, called the *lag period,* the bacteria begin to divide. In rich medium, a culture of a typical strain will double in number every 20 or 30 min. This phase of *exponential growth* of the cells in the culture is called *log phase* (sometimes subdivided into *early-log, middle-log,* and *late-log phases*). Eventually the cell density increases to a point at which nutrients or oxygen become depleted from the medium, or at which waste products (such as acids) from the cells have built up to a concentration that inhibits rapid growth. At this point, which under normal laboratory conditions occurs when the culture reaches a density of 1 to $2*10^9$ cells/ml, the cells stop dividing rapidly. This phase is called *saturation*, and a culture that has just reached this density is said to be freshly saturated.

MEDIA PREPARATION

Recipes are provided below for minimal liquid media, rich liquid media, solid media, top agar, and stab agar. Tryptone, yeast extract, agar (Bacto-agar), nutrient broth, and Casamino Acids are from Difco. NZ Amine A is from Hunko Sheffield (Kraft).

MINIMAL MEDIA

Ingredients for these media should be added to water in a 2-liter flask and heated with stirring until dissolved. The medium should then be poured into separate bottles with loosened caps and autoclaved 15 min at 15 lb/in^2, 121°C. Do not add nutritional supplements or antibiotics to any medium until it has cooled to >50°C. After the bottles cool to below 40°C, the caps can be tightened and the concentrated medium stored indefinitely at room temperature.

M9 medium, 5×, *per liter*
30 g Na_2HPO_4
15 g KH_2PO_4
5 g NH_4Cl
2.5 g NaCl
15 mg $CaCl_2$ (optional)

M63 medium, 5×
Per liter:
10 g $(NH_4)_2SO_4$
68 g KH_2PO_4
2.5 mg $FeSO_4*7H_2O$
Adjust to pH 7 with KOH

A medium, 5×
Per liter:
5 g $(NH_4)_2SO_4$
22.5 g KH_2PO_4
52.5 g K_2HPO_4
2.5 g sodium citrate$*2H_2O$

Before they are used, concentrated media should be diluted to 1× with sterile water and the following sterile solutions added, per liter:

1 ml 1 M $MgSO_4*7H_2O$
10 ml 20% carbon source (sugar or glycerol)

and, if required:

0.1 ml 0.5% vitamin B1 (thiamine)
5 ml 20% Casamino Acids *or*
L amino acids to 40 µg/ml *or*
DL amino acids to 80 µg/ml

RICH MEDIA

Unless otherwise specified, rich media should be autoclaved for 25 min. Antibiotics and nutritional supplements should be added only after the solution has cooled to 50°C or below. A flask containing liquid at 50°C feels hot but can be held continuously in one's bare hands.

H medium, *per liter*
10 g tryptone
8 g NaCl

Lambda broth, *per liter*
10 g tryptone
2.5 g NaCl

LB medium, *per liter*
10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH

The original recipe for LB medium (sometimes referred to as Luria or Lenox broth), does not contain NaOH. There are many different recipes for LB that differ only in the amount of NaOH added. Even though the pH is adjusted to near 7 with NaOH, the medium is not very highly buffered, and the pH of a culture growing in it drops as it nears saturation.

NZC broth, *per liter*
10 g NZ Amine A
5 g NaCl
2 g $MgCl_2*6H_2O$
Autoclave 30 min at 15 lb/in_2, 121°C. Cool to <50°C and add 5 ml 20% Casamino Acids.

Superbroth, *per liter*
32 g tryptone
20 g yeast extract
5 g NaCl

5 ml 1 N NaOH

TB (terrific broth), *per liter*
12 g Bacto tryptone
24 g Bacto yeast extract
4 ml glycerol
Add H_2O to 900 ml and autoclave, then add to above sterile solution 100 ml of a sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4.

Tryptone broth, *per liter*
10 g tryptone
5 g NaCl

2× TY medium, *per liter*
16 g tryptone
10 g yeast extract
5 g NaCl

TYGPN medium, *per liter*
20 g tryptone
10 g yeast extract
10 ml 80% glycerol
5 g Na_2HPO_4
10 g KNO_3

SOLID MEDIA

Liquid media can be solidified with agar. For minimal plates, dissolve the agar in water and autoclave separately from the minimal medium; autoclaving the two together will give rise to an insoluble precipitate. For rich plates, autoclave the agar together with the other ingredients of the medium. Cool the agar to about 50°C and add other ingredients if necessary. At this temperature, the medium will stay liquid indefinitely, but it will rapidly solidify if its temperature falls much below 45°C. Finally, pour the medium into sterile disposable petri dishes (*plates*) and allow to solidify.

Freshly poured plates are wet and unable to absorb liquid spread onto them. Moreover, plates that are even slightly wet tend to exude moisture underneath bacteria streaked on them, which can cause the freshly streaked bacteria to float away. So for most applications, dry the plates by leaving them out at room temperature for 2 or 3 days, or by leaving them with the lids off for 30 min in a 37°C

incubator or in a laminar flow hood. Store dry plates at 4°C, wrapped in the original bags used to package the empty plates.

Minimal Plates

Autoclave 15 g agar in 800 ml water for 15 min. Add sterile concentrated minimal medium and carbon source. After medium has cooled to about 50°C, add supplements and antibiotics. Pouring 32 to 40 ml medium into each plate, expect about 25 to 30 plates per liter.

Rich Plates

To ingredients listed below, add water to 1 liter and autoclave 25 min. Pour LB and H plates with 32 to 40 ml medium, in order to get 25 to 30 plates per liter. Pour lambda plates with about 45 ml medium for about 20 plates per liter.

H plates, per liter
10 g tryptone
8 g NaCl
15 g agar

Lambda plates, per liter
10 g tryptone
2.5 g NaCl
10 g agar

LB plates, per liter
10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
15 g agar or agarose

Additives
Antibiotics (if required):
Ampicillin to 50 µg/ml
Tetracycline to 12 µg/ml
Other antibiotics, see Table A.3E.1
Galactosides (if required):
Xgal to 20 µg/ml
IPTG to 0.1 mM

Estimating Viable Bacteria Numbers by Plate Counts (Plating method)

1. Make 10-fold serial dilutions in PBS:
   Prepare sterile 1.5 ml tubes with 0.9 ml PBS in each. *A good starting range is to plate 0.1 ml of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions. It is desirable to have between 30 and 300 colonies on the plate for calculations. Based on the OD600 reading you can estimate the range of dilutions necessary. An OD600 of 0.5 gives approximately 10^{8} bacteria per ml. Therefore, 0.1 ml of a 10^{-5} dilution should give about 100 colonies on a plate.*

2. Prepare agar plates with sterile glass beads (4 to 6 each). If possible, make two plates for each dilution. This is required when an accurate estimation of viable bacteria per ml is needed.

3. Make serial dilutions starting with 10^{-1} as far as needed:
   So pipet 0.1 ml sample into a tube of 0.9 ml PBS.
   Vortex well.
   Change the pipet tip.
   Pipet 0.1 ml of this into another tube of 0.9 ml PBS and vortex. Continue similarly until all dilutions are made.

4. Plate 0.1 ml of each dilution (starting with the 10^{-3}) on duplicate plates (The "real" dilution factor is therefore 10^{-4}). Shake plates to allow the beads to roll and spread the bacterial suspension.

5. Deposit the beads in a beaker containing soapy water.

6. Incubate all plates in the inverted position at the appropriate temperature until colonies appear (usually 1 to 2 nights).

7. Count colonies on a plate containing between 30 and 300 colonies (or an average of duplicate plates) and calculate the **number of bacteria per ml** using the following formula:

Colonies on plate X Dilution factor X 10 (because only 0.1 ml was plated)

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[a] Date assembled from **Karen Lech and Roger Brent.** *Current Protocols in Cytometry (2000) Appendix 3E-1-Appendix 3E-8*

ATTACHMENT 4. Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies[b]

Microfluidic systems provide a powerful platform for biological assays. In microfluidics, small volumes of solvent, sample, and reagents are moved through microchannels embedded in a chip. Examples of bioassays and biological procedures that have been miniaturized into a chip format include DNA sequencing, polymerase chain reaction (PCR), electrophoresis, DNA separation, enzymatic assays, immunoassays, cell counting, cell sorting, and cell culture. Miniaturized versions of bioassays offer many advantages, including small requirements for solvents, reagents, and cells (critical for valuable samples and for high-throughput screening), short reaction times, portability, low cost, low consumption of power, versatility in design, and potential for parallel operation and for integration with other miniaturized devices.

PDMS: properties and fabrication

The use of PDMS elastomer for miniaturized bioassays has numerous advantages over silicon and glass. PDMS as a material is inexpensive, flexible, and optically transparent down to 230 nm (and therefore compatible with many optical methods for detection). It is compatible with biological studies because it is impermeable to water, nontoxic to cells, and permeable to gases. A final, major advantage of PDMS over glass and silicon is the ease with which it can be fabricated and bonded to other surfaces. For the development of bioassays, where many designs may need to be tested, the ease of rapid prototyping in PDMS is a critical advantage.

Procedures for the fabrication of PDMS structures for microfluidics have been described in detail elsewhere. Briefly, the design of the microstructures is made in a computer-aided design (CAD) program. Using commercial services, the CAD-generated patterns are printed on transparencies (these services have overnight turnaround times). Lateral resolutions of 25 mm can be routinely achieved with image setters operating at 5080 dots per inch, and can be extended to 8 mm using photoplotters operating at 20 000 dots per inch [10]. (For features beyond 8 mm,

chrome masks can be used, but they take longer to fabricate commercially, and are more expensive than transparencies.) The transparency is then used as a photomask in UV-photolithography to generate a master. In this procedure, a thin layer of photoresist (for example, the photocurable epoxy SU-8) is spin-coated onto a silicon wafer. Using different types of SU-8 of various viscosities, thicknesses of 1–300 mm can be reliably spin-coated. The photoresist is exposed to UV light through the photomask, and a developing reagent is used to dissolve the unexposed regions. The resulting bas-relief structure serves as a master for fabricating PDMS molds.

To create the PDMS mold, the surface of the silicon/ photo-resist master is treated with fluorinated silanes (which prevents irreversible bonding to PDMS), and a liquid PDMS prepolymer (in a mixture of 1:10 base polymer: curing agent) is poured onto it. The PDMS is cured at 707C for 1 h or more and peeled off the master, producing the final replica bearing the designed microstructures. Small holes are drilled into the PDMS using a borer to produce inlets and outlets. Finally, PDMS can seal to itself and other flat surfaces reversibly by conformal contact (*via* van der Waals forces), or irreversibly if both surfaces are Si-based materials and have been oxidized by plasma before contact (a process that forms a covalent O-Si-O bond). Seals are watertight and can be formed under ambient conditions (unlike silicon and glass, for which C bonding requires high temperatures or adhesives). If desired, many PDMS replicas can be made from a single master. This procedure of producing the PDMS structure from the silicon master, called replica molding, can be carried out under normal laboratory conditions without an expensive clean room, and can replicate certain types of features with dimensions down to 10 nm. Replica molding, along with procedures such as microcontact printing, casting, injection molding and embossing, comprise a set of techniques for manipulating elastomeric structures called soft lithography. Although we focus on PDMS in this review, soft lithography has been demonstrated for other elastomers (such as polyurethane and epoxy).

PDMS consists of repeating -OSi(CH3)2- units; the CH3 groups make its surface hydrophobic. This hydrophobicity results in poor wettability with aqueous solvents, renders microchannels susceptible to the trapping of air bubbles, and makes the

surface prone to nonspecific adsorption to proteins and cells. The surface can be made hydrophilic by exposure to an air plasma (in a plasma cleaner for 1 min); the plasma oxidizes the surface to silanol (Si-OH). The plasma-oxidized surface remains hydrophilic if it stays in contact with water. In air, rearrangements occur within 30 min, which bring hydrophobic groups to the surface to lower the surface free energy. The surface of oxidized PDMS can be modified further by treatment with functionalized silanes.

Figure 15. Design of simple micro-channel device used in our preliminary Biofilm experiments.

FIGURE TAKEN FROM THE AUTHOR

[b] Information taken from Samuel K. Sia and George M. Whitesides (32)

Table 1. Antibiotics, Their Modes of Action, and Modes of Bacterial Resistance[a]

Antibiotic [b]	Stock conc. (mg/ml)	Final conc. (µg/ml)	Mode of action	Mode of resistance
Ampicillin[c]	**4**	**50**	Bacteriocidal; only kills growing *E. coli*; inhibits cell wall synthesis by inhibiting formation of the peptidoglycan cross-link	β-lactamase hydrolyzes ampicillin before it enters the cell
Chloramphenicol in methanol	**10**	**20**	Bacteriostatic; inhibits protein synthesis by interacting with the 50S ribosomal subunit and inhibiting the peptidyltransferase reaction	Chloramphenicol acetyltransferase inactivates chloramphenicol
D-Cycloserine,[d] in 0.1 M sodium phosphate buffer, pH 8	**10**	**200**	Bacteriocidal; only kills growing *E. coli;* inhibits cell wall synthesis by preventing formation of D-alanine from L-alanine and formation of peptide bonds involving D-alanine	Mutations destroy the D-alanine transport system
Gentamycin	**10**	**15**	Bacteriocidal; inhibits protein synthesis by binding to the L6 protein of the 50S ribosomal subunit	Aminoglycoside acetyltransferase and aminoglycoside nucleotidyltransferase inactivate gentamycin; mutations in *rplF* (encodes the L6 protein) prevent the gentamycin from binding
Kanamycin	**10**	**30**	Bacteriocidal; inhibits protein synthesis; inhibits translocation and elicits miscoding	Aminoglycoside phosphotransferase, also known as neomycin phosphotransferase, aminoglycoside acetyltransferase, and aminoglycoside nucleotidyltransferase; inactivates kanamycin
Kasugamycin	**10**	**1000**	Bacteriocidal; inhibits protein synthesis by altering the methylation of the 16S RNA and thus an altered 30S ribosomal subunit	Mutations prevent kasugamycin from binding to the ribosome; mutations decrease uptake of kasugamycin
Nalidixic acid, pH to 11with NaOH	**5**	**15**	Bacteriostatic; inhibits DNA synthesis by inhibiting DNA gyrase Mutations in the host DNA gyrase prevent	Mutations in the host DNA gyrase prevent nalidixic acid from binding
Rifampicin,[e] in methanol	**34**	**150**	Bacteriostatic; inhibits RNA synthesis by binding to and inhibiting the β subunit of RNA polymerase; rifampicin sensitivity is dominant.	Mutations in the host DNA gyrase prevent nalidixic acid from binding
Spectinomycin	**10**	**100**	Bacteriostatic; inhibits translocation of peptidyl tRNA from the A site to the P site	Mutations in *rpsE* (encodes the S5 protein) prevent spectinomycin from binding; spectinomycin sensitivity is dominant and resistance is recessive
Streptomycin	**50**	**30**	Bacteriocidal; inhibits protein synthesis by binding to the S12	Aminoglycoside phosphotransferase

			protein of the 30S ribosomal subunit and inhibiting proper translation; streptomycin sensitivity is dominant	inactivates streptomycin; mutations in *rpsL* (encodes the S12 protein) prevent streptomycin from binding; streptomycin resistance is recessive
Tetracycline,[e] in 70 % ethanol	**12**	**12**	Bacteriostatic; inhibits protein synthesis by preventing binding of aminoacyl tRNA to the ribosome A site	Active efflux of drug from cell

[a] Date assembled from **Karen Lech and Roger Brent.** *Current Protocols in Cytometry (2000) Appendix 3E-1-Appendix 3E-8*

[b] All antibiotics should be stored at 4°C, except tetracycline, which should be stored at −20°C. All antibiotics should be dissolved in sterile distilled H_2O unless otherwise indicated.

[c] Carbenicillin, at the same concentration, can be used in place of ampicillin. Carbenicillin can be stored in 50% ethanol/50% water at −20°C.

[d] D-cycloserine solutions are unstable. They should be made immediately before use.

Table 2. **Proteins and genes involved in pathogenesis of EHEC**

Genetic Locus	Protein description	Gene(s)	Function
Chromosome (locus of enterocytes effacement)	Intimin Tir Secretion proteins Type III secretion system	*eae* *tir* *espA, espB, espD* *escC, escD, escF, escJ, escN, escR, escS, escT, escU, escV, sepQ, sepZ*	Adherence Intimin receptor Induces signal transduction Apparatus for extracellular protein secretion
Phage	Stx	*stx1, stx2, stx2c, stx2d*	Inhibits protein synthesis
Plasmid	EHEC hemolysin Catalase-peroxidase	EHEC-*hylA* *katP*	Disrupts cell membrane permeability