Effect of silver coated carbon nanotubes on metabolically essential genes expression and outer membrane protein profile of *Escherichia coli*

Atul A Chaudhari, Shree R. Singh, Shreekumar Pillai*

* Center for Nanobiotechnology Research, Alabama State University, Montgomery, AL-36104,
spillai@alasu.edu, atulvet@gmail.com, ssingh@alasu.edu

ABSTRACT

In this growing world, antibiotic resistance of food borne pathogens such as *E. coli* is a global concern. Recently, use of nanoparticles has been shown to be an effective alternative to antibiotics. Specifically, Silver coated carbon nanotubes (AgCNTs) have been shown to possess strong antibacterial activity against several multi drug resistant pathogens. It is however extremely important to reveal the exact molecular mechanism for their antibacterial activity. In the present study, we investigated the effect of AgCNTs on metabolically important gene's expression and outer membrane proteins of *Escherichia coli*. Bacterial cells were treated with 10 µg/ml of AgCNT for 16h and qRT-PCR was performed to investigate the expression of genes associated with amino acid biosynthesis (*argC*, *metL*, *metR*), DNA binding transcriptional activators (*csgD* and *rstA*), DNA repair (*mfd*, *recN*, *ycaJ*), nucleic acid metabolism (*carB*, *purM*, *nrdF*) and TCA cycle (*aceF*, *frdB*). Also, the outer membrane proteins (OMPs) were extracted and the samples were run using Experion Pro260 gel. Our results showed that metabolically essential gene's expression was significantly upregulated several folds (~4-5 folds) in AgCNTs-treated *E. coli* compared to untreated bacteria. Also, the OMP pattern in AgCNTs-treated bacteria showed that OMPs were either missing or downregulated in treated bacteria compared to non-treated bacteria. Our results thus indicate that antibacterial activity of AgCNTs can be attributed to its ability to damage the outer membrane protein structure of bacteria and qRTPCR results are indicative of molecular mechanism for antibacterial effect that are characterized by DNA damage and nucleic acid metabolism of bacteria.

Keywords: silver coated carbon nanotubes, mechanism, metabolic, gene, and protein

1 INTRODUCTION

Antibiotic resistance is a global concern as far as the food borne bacterial pathogens are concerend [1]. Therefore suitable alternatives are needed to treat the infections associated with the multi drug resistant bacteria. Nanoparticles and their metallic composites are gaining popularity due to their antimicrobial properties [2,3]. In particular, silver coated carbon nanotubes (AgCNTs) have been reported as effective antimicrobials [4,5].

Despite of their antibacterial properties, very less is known about the mechanism for the antibacterial action of AgCNTs. Some of the reported mechanisms include silver ion dissolution and cell membranes damage via generation of reactive oxygen species (ROS) through direct contact of AgCNTs with bacterial cells [5]. However, the exact molecular mechanism of action is yet to be defined clearly. Expression of genes associated with normal physiological process of bacteria are essential for maintaining the membrane integrity, DNA damage, DNA repair, transcriptional activation etc. The expression of the genes assocaited with the above functions get highly affected due to the stress conditions. The stress conditions include either change in the extracellular enviornment or interaction with a drug, specifically an antibiotic. Depending upon the severity of the change, expression of these genes are affected significantly. If the damage occurs to the outer membrane protein assembly, the outer membrane proteins are produced in high quantities to recover the damage to the membranes. Generally the expression of genes are designated either up-regulated or down regulated in response to the external stimuli. Upregulation of the genes indicate the stress response and down regulation of the expression indicate the severity of the damage. Also, gene expression eventually affects the protein expression levels. There are several putative and well known genes that encode for DNA repair, DNA damage, activation of transcription, amino acid biosynthesis etc. Therefore, it would be appropriate to investigate the expression of these genes in bacteria exposed to AgCNTs.

Accordingly, the present study was designed to investigate the effects of AgCNTs on expression of genes that are associated with the normal physiological processes in *Escherichia coli*, a gram negative food borne pathogen. The expression of the genes associated with amino acid biosynthesis (*argC*, *metL*, *metR*), DNA binding transcriptional activators (*csgD* and *rstA*), DNA repair (*mfd*, *recN*, *ycaJ*), nucleic acid metabolism (*carB*, *purM*, *nrdF*) and TCA cycle (*aceF*, *frdB*) were investigated using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The outer membrane proteins (OMPs) were extracted as descrined eariler [6] the samples were run using Experion Pro260 gel.

2 MATERIALS AND METHODS

2.1 Preperation of AgCNTs suspension

1-5 nm sized AgCNTs (95% pure), were purchased from NanoLab, Inc. (Waltham, MA, USA). 1mg of AgCNTs were suspended in 1ml of water, sonicated for 1-2h to obtain 1mg/ml suspension.

2.2 Bacterial growth conditions

Escherichia coli (ATCC® 25922™) was purchased from American Type Culture Collection (ATCC, VA USA). Bacteria were grown at 37°C in Luria-Bertani (LB) broth (Difco, Sparks, MD, USA) For all the experiments, *Escherichia coli* was treated with 10 µg/mL of AgCNTs for 16h and compared with the untreated controls.

2.3 qRT-PCR

The mRNA levels of genes of *Escherichia coli* were investigated using qRT-PCR. The oligonucleotide pairs are described in Table 1. The bacteria (1 × 105 cfu/ml) were treated with 10 µg/mL of AgCNTs for 16h in a shaking incubator (250 rpm) at 37 °C, followed by total RNA extraction using RNeasy Mini kit (Qiagen, Germany). DNA was further amplified and quantified in the Applied Biosystems® ViiA™ 7 real time PCR system (Life Technologies) using SYBR® Select Mastermix (Life Technologies, Grand Island, NY). The PCR conditions used were: initial denaturation at 95 °C for 5 mins, followed by 40 cycles of 95 °C for 5 sec, 56 °C annealing temperature for 25 sec, extension at 72 °C for 30 sec and; the final 10 min extension at 72 °C

2.4 Extraction of outer membrane proteins (OMPs)

The OMP extraction was performed by a modified lysozyme-osmotic shock method as described previously [6]. Briefly, *Escherichia coli* cultures (untreated or tretaed with 10 µg/mL of AgCNTs for 16h) were grown in LB broth, centrifuged at 7,000 × *g* for 10 min. The cell pellets were lyzed in 100 mM Tris-HCl buffer (pH 8.6) containing 500 mM sucrose and 0.5 mM EDTA, followed by lysozyme addition. Further, 50 mM Tris-HCl buffer (pH 8.6) containing 250 mM sucrose, 0.25 mM EDTA, and 2.5 mM $MgCl_2$ were added and the mixture was incubated for 15 min on ice. Cells were centrifuged at 7,000 × *g* for 6 min and pellets were resuspended in 20 mM Tris-HCl (pH 8.6) and sonicated until the suspension appeared transparent. The lysates were centrifuged at 7,000 × *g* at 4°C for 6 min and the supernatant then was subjected to ultricentrifugation at 132,000 × *g* at 4°C for 1 h. The pellets were suspended in 4 ml of 20 mM Tris-HCl (pH 8.6) containing 1% Sarkosyl and incubated for 30 min on ice to isolate the outer membrane fraction. OMPs were collected upon centrifugation at 132,000 × *g* at 4°C for 1 h.

2.5 Measurement of protein concentraion

OMP concentration was measured by BCA™ protein assay kit (Thermo scientific, Rockford, IL, USA) as per the manufacturers instructions.

2.6 Electrophoresis using Experion Pro260

The OMPs pattern in the treated and non-treated bacteria was investigated using Experion Pro260 kit. The samples (100ng) were analyzed using The ExperionTM Pro260 (Bio rad, USA) automated electrophoresis system which uses LabChip microfluidic technology to automate protein electrophoresis for separation, detection and data analysis.

3 RESULTS AND DISCUSSION

3.1 Expression of genes associated with amino acid biosynthesis

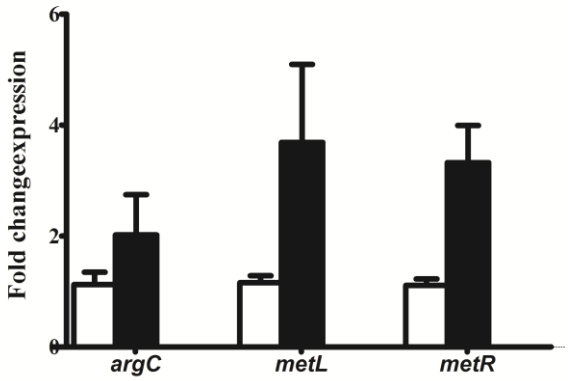

Figure 1: **q**RT-PCR analysis of genes associated with amino acid biosynthesis such as *argC*, *metL* and *metR*. Empty bars-untreated; Black color bars-treated with AgCNTs

The expression of genes encoding for the biosynthesis of two amino acids such as arginine (*argC*) and methionine (*metR* and *metL*) were significantly upregulated compared to untreated bacteria (Figure 1). Upregulation of these genes indicated that the amino acid biosynthesis machinary is significantly affected in AgCNTs treated bacteria. In all, it indicated the stress response induced by AgCNTs treatment.

3.2 DNA binding transcription activators

Genes associated with DNA binding to activate the transcription process such as *csgD* and *rstA* were significantly up regulated upon AgCNTs exposure (Figure

2). This indicated that AgCNTs interupt the genes associated with DNA binding.

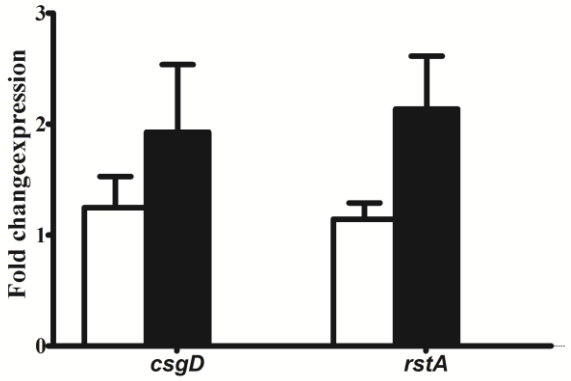

Figure 2: DNA binding transcription activators. Genes such as *csgD* and *rstA* regulating DNA binding and activation of transcription

Besides the well defined contact mediated mechanism for antibacterial activity of AgCNTs [5], our results indicate that the antibacterial activity can also be associated with the regulation of DNA transcription activators.

3.3 Expression of genes involved in DNA repair

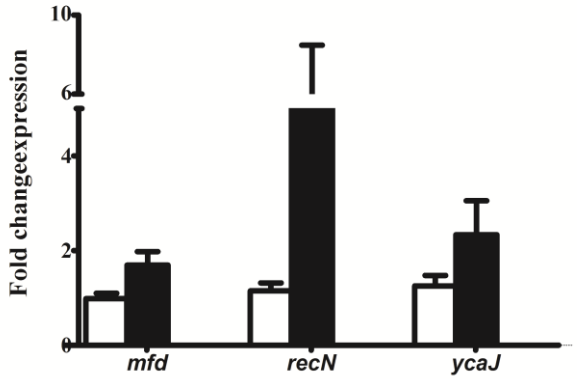

Figure 3: Gene expression of genes such as *mfd, recN* and *ycaJ*, involved in DNA repair

When exposed to 10 µg/ml of AgCNTs, the gens associated with DNA repair were upo regulated several folds in *Escherichia coli*. In particular, *recN* gene was upregulated almost 6 folds compared to untreated bacteira. This result indicated that AgCNTs regulate their antibacterial activity by causing damage directly to the DNA of bacteria. The upregulation of these genes only occur when there is a significant damage to DNA in response to the external stimuli and to repair the damage. Our results thus clearly showed that besides regulating the gene expression associated with metabolism of bacterial cells, AgCNTs also causes damage to the bacterial DNA and attributes to the antibacterial activity of AgCNTs.

3.4 Genes associated with Nucleic acid synthesis

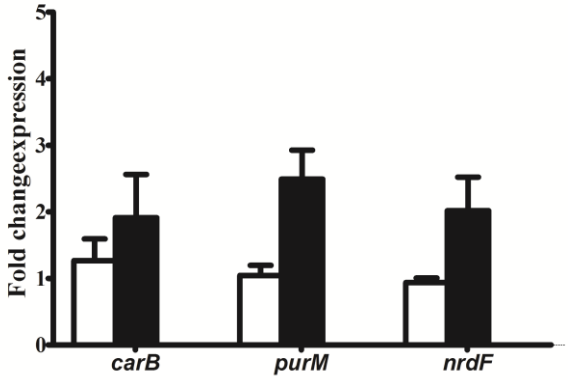

Figure 4: qRT-PCR analysis of the genes associated with nucleic acid synthesis

We further investigated the expression of genes associated with the nucleic acid synthesis such as *carB, purM* and *nrdF*. The treated bacteria exhibited a significant upregulation of these genes compare to the untreated bacteria (Figure 4). In concurrent with the upregulation of genes associated with DNA repair, nucleic acid synthesis also appears to be affected by AgCNTs exposure. Our result thus clearly indicate that affecting nucleic acid synthesis could also be one of the molecular mechanisms involved in the antibacterial activity of AgCNTs.

3.5 TCA cycle associated genes

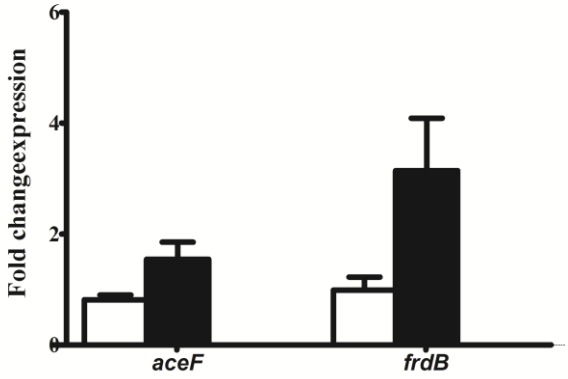

Figure 5: Expression of the genes associated with TCA cycle

Similarly, the genes associated with the TCA cycle which is a metabolic pathway were upregulated (2-3 folds) in bacteria treated with AgCNTs (Figure 5).

3.6 OMP pattern

The OMP pattern of the bacteria exposed to AgCNTs was also investigated and compared with the untreated control bacteria.

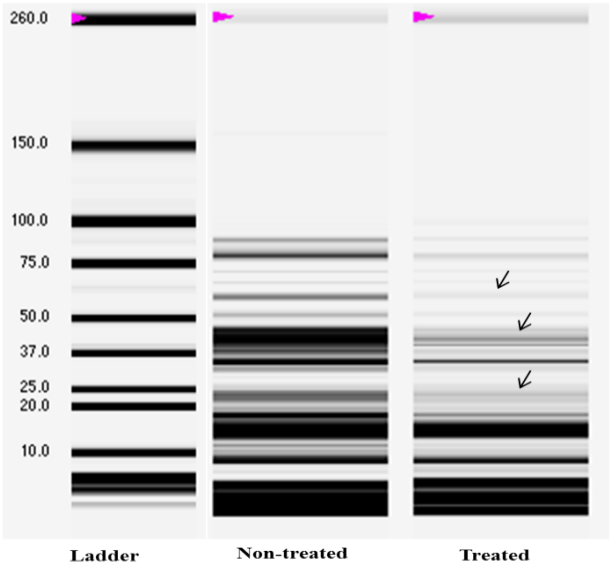

Figure 6: OMP pattern of *Escherichia coli.*Arrow indicate the proteins whose expression was less comapred to non-treated bacteria

As shown in figure 6, OMP pattern of the bacteria treated with AgCNTs showed significant difference when compared with the untreated bacteria.

Table 1. Oligonucleotides used in this study.

Gene	Forward (5`-3`)
recN	TCTCGATCCCAACCGACTAT AGGGCTGACGTGATGTTTAC
carB	CTGCATCATCGTCTGCTCTATC TCTTTGTCGGTCAGCGTTT
purM	CGACTATTACGCAACCGGAAA ACCAGTGAACAGCCTGATTG
nrdF	CTACGCCTGGAGTGAAGAAA CAGCCAGAAACCGGAATAGA
mfd	TCAGGAAGCTGGAAGGTAATG GGACCATCAAGGCGGTAAT
ycaJ	GTACTAACTCAGGCGATGGAAG CCATTCACCAGTTCAGCAATG
argC	CCTGGCAACCAGACATAAGA CATATGCGGATGGCGATTTAC
metL	CGACAGCGGCGATACTATTT GATCCACCAGCTCGGTAAAC
metR	GGAGAGCTGGATCTGGTAATG ACACCAGACGCACTTCATAG
rstA	CTCGATAGCGATATGAACCACATCCTGG GTAGGGAGTCAGAGACGTTTCCTGAATAC
csgD	GTTGTTTTTCCTGCTCAAAGTATCCTGCC ACTAAACCTTCTTTGCAGGCGACAGCTC
frdB	CACGGTAAGAAGGAGCGTATG TCTACTTTGCCCTGCTGAATG
aceF	CAGATGCCTCGCTTCAATAGT CAGCTCGATGATGCCTTTCT
16s rRNA	GACTCCTACGGGAGGCAGCA CAGCCATGCAGCACCTGTCT

Some of the bands were either missing or were less expressed (indicated by arrow). It suggest that AgCNTs react with the outer membrane. These findings support the contact mediated antibacterial effect of AgCNTs as described previously [5]. Our results here further demonstrate that the outer membranes of the bacteria are damaged as evidenced by the different outer membrane proteins profile.

In conclusion, our results indicate that antibacterial activity of AgCNTs can be attributed to its ability to damage the outer membrane protein structure of bacteria and qRTPCR results are indicative of molecular mechanism for antibacterial effect that is characterized by DNA damage and nucleic acid metabolism of bacteria.

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