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Amplification of ndvB gene and biofilm formation studies in *Escherichia coli*

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ABSTRACT

Biofilms are complex microbial communities that grow at interfaces, often at solid-liquid surfaces. Biofilms are usually found on solid substrates submerged in or exposed to some aqueous solution, although they can form as floating mats on liquid surfaces and also on the surface of leaves, particularly in high humidity climates. The main objective of the present study was isolation, purification, and characterization of E.coli. Genomic DNA was extracted from the identified isolate, and analyzed using biochemical characterization. The aim of the present study was to understand the presence of ndvB gene in E.coli which in turn is responsible for antibiotic resistance through biofilm formation. E.coli was isolated for biofilm forming regions. The selected E.coli colonies were sub cultured and purified on EMB Agar plates and were further maintained on Nutrient Agar slants. For further confirmation the selected E.coli strains were morphologically characterized by Gram's staining and were found to be gram negative, short rods. They were further biochemically characterized by tests such as Indole test, Methyl Red Test, VP test and Citrate test. All the strains were positive for Indole test and Methyl Red test, Voges Proskaeur test and Citrate utilisation test were negative. Hence from these tests it was further confirmed that the isolated strains belonged to the genus Escherichia coli. The ndvB gene was amplified and the biofilm studies were confirmed by ethanol method and primary adherence test. The biofilm assay and the primary adherence assay strongly suggests of the biofilm forming nature of the E.coli.

Keywords: *E. coli*, ndvB gene, Biofilm formation assay, Primary adherence assay.

INTRODUCTION

Biofilm development is not a simple and uniform process, but rather is more complex and differentiated than it appears on the surface [1]. Biofilm formation proceeds as a controlled developmental sequence, and five stages have been suggested. In stages one and two, a temporary association with the surface through weak chemical interactions, such as van der Waals forces, occurs followed by strong adhesion [2]. The surface appendages such as pili, fimbriae and curli as well as the production of the EPS participate in this stage. Stages three and four include the aggregation of cells into microcolonies with consequent growth and maturation. During the maturation of the biofilm, the cells continue to grow and produce more EPS, causing an expansion of the microcolonies [3]. These large microcolonies then merge forming even thicker intricate architectures that include regions filled with water. Stage five is characterized by a return to transitory motility where biofilm cells are shed and disseminated either individually or in groups into the surrounding environment with a return to the planktonic state [4].

Biofilm structures can be flat or mushroom-shaped depending on the nutrient source, and its structure can change according to nutrient conditions, surface and interface properties and hydrodynamics. For example, biofilms formed in running waters are filamentous and patchy, biofilms in quiescent waters have mushroom or mound like structures [6,7]. Biofilms under sheer stress are stratified and compacted, biofilms from sludge reactors are aggregative.

Escherichia coli biofilm initiation and maturation can involve many diverse factors and its capability to form a biofilm depends significantly on environmental circumstances [8,9,10]. Even well-demonstrated adhesion factors can be exchanged by others in some cases. Numerous different pathways can be used during E. coli biofilm formation, and several regulatory mechanisms could coordinate the biofilm adhesion and maturation processes [11]. In E. coli, motility has shown to have a link with its ability to form a biofilm since its flagella allows bacteria to spread along the surface. However, it is not a requirement, and nonmotile bacteria can still form biofilms under certain conditions where the expression of robust adhesion factors may substitute for force generating movements during the initial interactions between adhering bacteria and the surface [12].

Biofilm formation occurs by at least three different mechanisms. In one mechanism, type IV pili-mediated twitching motility encourages surface aggregation. Alternatively, attached cells spread outward and upward by binary division to form cell clusters, or cells are recruited from the bulk fluid to form of biofilms. The relative contribution of these mechanisms depends on the organisms, the nature of the surface, and the physical – chemical condition of the environment [13]. The twitching motility, growth rate, cell signalling, exopolysaccharide production, and the physical growth environment all play a significant role in the biofilm structure. Bacteria growing in biofilms are responsible for a large number of persistent infections and are often more resistant to antibiotics than are free-floating bacteria [14,15]. Biofilms offer a mode of growth for bacteria that allow them to survive and indeed thrive in a host. The survival of bacteria exposed to toxic compounds is a multifactorial phenomenon, involving well-known molecular mechanisms of resistance but also less-well-understood mechanisms of tolerance that need to be clarified. In particular, the contribution of biofilm formation to survival in the presence of toxic compounds, such as nickel, was investigated in this study [16,17].

E. coli is nonspore-forming and beta hemolytic. On MacConkey Agar, it usually ferments lactose or produce pink colonies with surrounding areas of precipitated bile salts. It also presents with a green sheen on eosin methylene blue agar. E. coli strain will produce indole from tryptophan; it does not produce hydrogen sulfide, urease, and cannot use citrate as sole carbon source [20]. Pathogenic strains of E. coli are responsible for three types of infections in humans; urinary tracts infections, neonatal meningitis, and intestinal diseases. Uropathogenic E. coli (PEC) causes 90 % of urinary tract infections in anatomically normal and unobstructed urinary tracts. The uropathogenic strains have an adherence factor called P fimbriae, or pili, which binds to the P blood group antigen and mediates the attachment of E. coli to uroepithelial cells. Thus, patients with intestinal carriage of this strain are at greater risk of developing UTI than the general population [22].

One of these genes, *ndvB*, encodes a glucosyltransferase involved in the formation of cyclic glucans. The glucans are cyclic polymers of 12 to 15 β-(1→3) linked glucose molecules with phosphoglycerol substitutions. Inactivation of *ndvB* blocked glucan production but did not affect growth, the kinetics of biofilm formation, or the architecture of the biofilms [12,16].

Expression of *erbR* in Δ*ndvB* biofilms was restored after the treatment of the biofilm with periplasmic extracts derived from wild-type biofilm cells. Inactivation of ethanol oxidation genes increased the sensitivity of biofilms to tobramycin [15,2]. Together, these results reveal that *ndvB* affects the expression of multiple genes in biofilms and that ethanol oxidation genes are linked to biofilm-specific antibiotic resistance [11].

The present study was undertaken to isolate the bacterial isolates from the contaminated kitchen waste. The isolates were screened and subcultured to isolate the genomic DNA. The extracted DNA was used for amplifying ndvB gene (Biofilm forming gene). The isolates were also tested for their ability to form the Biofilm and their primary adherence capacity.

MATERIALS AND METHODS

ISOLATION AND IDENTIFICATION: Contaminated water samples from the kitchen waste pipelines were collected in sterile centrifuge tubes. For the isolation of pathogenic *E.coli*, the samples were streaked unto the EMB agar plates and incubated at 37 ºC for 24 h. The colonies were isolated on the basis of the clear zone produced by the organism and colony morphology shape, size, structure, texture, appearance, elevation and colour. Further identification was done on the basis of staining and biochemical tests. Differential staining i.e. Gram staining was performed.

BIOCHEMICAL CHARACTERIZATION: The different isolates obtained were screened for grams staining. Different mediums were used for the biochemical characterization of the isolated and selected bacteria for their identification according to *Bergey's Manual of Determinative Bacteriology***.** Determination of the physiological properties of strains was performed according to the biochemical tests recommended. These tests included, among

other assays, aerobic or anaerobic growth, pH range of growth, test for motility, methyl-red and Voges–Proskauer tests, Oxidase, Catalase, hydrolysis of casein, citrate utilization, nitrate reduction, Indole production, Urease test, gelatin hydrolysis, and hydrolysis of polysaccharides and fermentation of various sugars.

Molecular Characterization of the isolates:

DNA isolation: The isolated colonies were then cultured in Luria-Bertani broth and incubated at 37°c for 48 hours. Following the incubation, 2ml bacterial culture was centrifuged at 6000 rpm for about 10 minutes. To the pellet 1ml of lysis buffer (10mM Tris HCl, pH 8; 0.5M EDTA; 0.5% SDS; 1M NaCl) was added and vortexed properly and incubated at 45 0C in boiling water bath for 10 minutes. Following incubation, 1ml of phenol: chloroform mixture (1:1) was added to the mixture and centrifuge at 10,000rpm for 10 minutes. The upper aqueous layer was transferred and equal volume of chloroform: isoamyl alcohol mixture (24:1) was added and then 1/10th volume of 3M sodium acetate was added. The contents are mixed properly and centrifuged at 10,000 rpm for 10 minutes. To the upper aqueous layer double the volume of chilled ethanol was added to precipitate the DNA and later centrifuged at 12,000 rpm for 10 minutes. The DNA pelleted was the stored in 20-50ml of TE buffer and stored at 4°C for further use. The extracted DNA was then quantified using the Nano drop spectrophotometer (ND-1000) to check for the purity. The pure DNA obtained thus obtained was run on 0.8% agarose gel to check for the DNA bands.

PCR amplification: The ndvB gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of ndvB gene. The forward primer for ndvB, 5′ GAGGTGGCAAAATGGGCAAG 3′ and the reverse primer, 5′- CATGCAGGCAAGAATCGACG 3′, were purchased from Eurofins, Bangalore. These primers correspond to the gene ndvB and thus the final PCR product was 781bp. The PCR mixture consisted of 10x reaction buffer with MgCl2 (1.5mM), 2µL of dNTP mix (2.5mM), 2µL each of forward and reverse primers (10picomoles/µl each primer), 0.3µL of Taq DNA polymerase (5 U/µL), and 50ng/µL of template DNA in a total volume of 20µL. The PCR was performed with the following cycling profile: initial denaturation at 94°C for 3 min, followed by 30 cycles of 30s denaturation at 94°C, annealing at 62°C for 30s, and extension at 72°C for 1min. The time for the final extension step was increased to 3 min. The PCR products amplified were then qualitatively analysed on 1% agarose gel.

Cultivation of biofilms: Biofilm formation studies were done by ethanol acetone method. Briefly, the overnight cultures from Luria Broth was taken and diluted to a ratio of 1: 200 using Luria Broth+Glucose solution. The contents are then transferred to Microtitre plate and incubated at 37°C for 24 hrs. After incubation the wells were washed 3 times with PBS (200µl in each well) and air dried. The wells were then stained with 2% crystal violet for about 15minutes and rinsed with tap water and air dried. The crystal violet was then solubilized in 200µl of ethanol:acetone 80:20 and the absorbance was measured at 590nm.

Primary adherence assay: The isolates were inoculated into Luria Broth containing 0.5% glucose and incubated at 37°C overnight. About 200µl of the broth with culture was diluted to an absorbance of 0.1 at 578nm with LB Broth containing 0.5% glucose. 10ml of this suspension was added to petri plates followed by incubation for two hours at 37°C. After incubation the petriplates were washed three times with PBS and the cells were then fixed with glycerine solution followed by gram staining. The adherent bacterial cells were observed under 40X and mean count was taken for 5 microscopic fields.

RESULTS

ISOLATION OF *Escherichia coli*: 10 *Escherichia coli* colonies were isolated from various biofilm samples. The strains were subcultured on Eosine Methylene Blue agar plates. *E.coli* cultures showed dark coloured colonies with green metallic sheen on EMB Agar. The selected isolates were subcultured and maintained on Nutrient Agar slants for further use. The isolated strains were morphologically characterized by Gram's Staining and were confirmed to be Gram negative and small rods.

Figure 1: *E.Coli* isolates subcultured on EMB Agar plates

Biochemical tests

Test	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Indole test	-	-	-	-	-	-	-	-	-	-
Methyl Red test	+	+	+	+	+	+	+	+	+	+
Voges Proskauer test	+	+	+	+	+	+	+	+	+	+
Citrate Utilization test	-	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+	+
Urease test	-	-	-	-	-	-	-	-	-	-
Nitrate reduction test	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis test	+	+	+	+	+	+	+	+	+	+
Caesin hydrolysis test	-	-	-	-	-	-	-	-	-	-

Table 1: Biochemical test results

DNA isolation: The qualitative analysis of DNA by gel electrophoresis is shown in following pictures. The obtained DNA showed sharp single bands on 0.8% agarose gel without any degradation or RNA contamination.

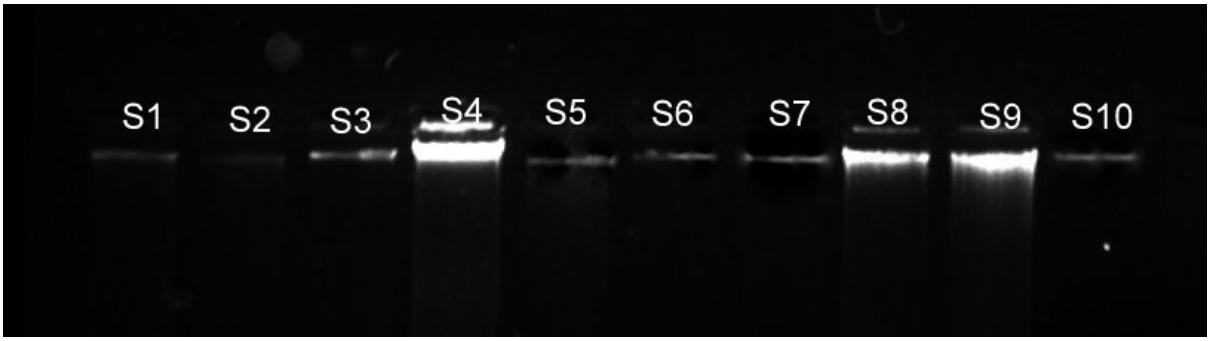

Fig 2: DNA isolation of the bacterial isolates. S1 to S10: Bacterial isolates

PCR amplification of ndvB gene: The samples were amplified with *ndvB* primers namely Forward primer: GAGGTGGCAAAATGGGCAAG; Reverse primer: CATGCAGGCAAGAATCGACG. The amplified product was run on a 1 % agarose gel. Sharp bands of band length of approximately 780bp were obtained. The amplified bands indicated the presence of ndvB gene in the selected *E. Coli* strains. Among the 10 isolates, the ndvB gene was found to be present in all of the selected isolates.

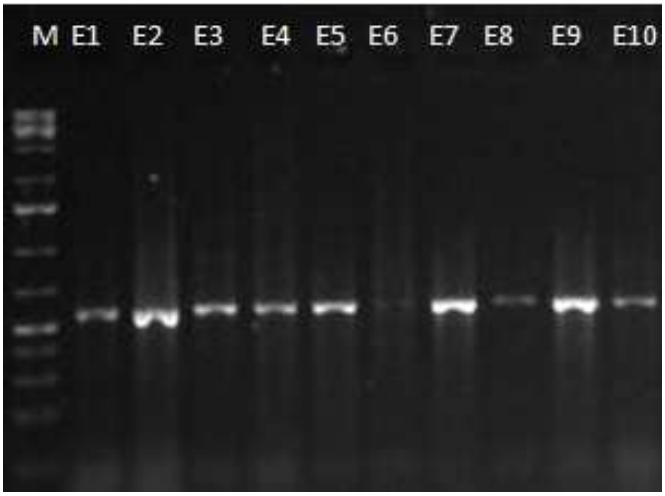

Fig 3 : PCR amplified products on 1% agarose gel. M: Molecular marker, E1, .E10: isolates. The band was found to be 780bp

Cultivation of Biofilms & Primary adherence assay: All the bacterial isolates were shown to processes the ability to form the biofilms. The values obtained were average of triplicates. The O.D value at 595nm showed the bacterial ability to form the strong biofilms. The negative control which was sterile distilled water added to the well showed an O.D value of 0.03, which is negligible. The bacterial isolates also showed positive response to the adherence assay. The cell count obtained proves of the capacity of the isolates to form strong ability to adhere to the wells. The values obtained were average of the count taken from five focus fields. The negative control which was distilled water showed a least count of 15-25 cells.

Table 2: Table showing the cell count of primary adherence assay of bacterial isolates and the O.D values of the Biofilm formation assay. All the values are the averages of triplicates. The cell count in adherence assay is the average of the counts taken from five focus fields

	1	2	3	4	5	6	7	8	9	10
Biofilm Assay	0.21 ± 0.21	0.22 ± 0.03	0.18 ± 0.07	0.183 ± 0.03	0.192 ± 0.01	0.203 ± 0.05	0.243 ± 0.05	0.232 ± 0.02	0.233 ± 0.07	0.213 ± 0.01
Adherence assay	756	744	654	687	673	599	634	651	432	867

DISCUSSION

The biofilms have evolved significantly increased antibiotic resistance relative to their free-floating counterparts, severely hampering the successful treatment of biofilm-associated infections. The increased antimicrobial resistance results from the simultaneous operation of multiple biofilm-specific mechanisms that are still not fully understood. Deeper understanding of these mechanisms will be useful for the development of new antibiofilm agents, from which innovative therapeutic measures may be developed to eradicate persistent infections. The aim of the present study was to understand the presence of ndvB gene in *E.coli* which in turn is responsible for antibiotic resistance through biofilm formation. E.coli was isolated for biofilm forming regions. The selected *E.coli* colonies were sub cultured and purified on EMB Agar plates and were further maintained on Nutrient Agar slants. For further confirmation the selected *E.coli* strains were morphologically characterized by Gram's staining and were found to be gram negative, short rods. They were further biochemically characterized by tests such as Indole test, Methyl Red Test, VP test and Citrate test. All the strains were positive for Indole test and Methyl Red test, Voges Proskaeur test and Citrate utilisation test were negative. Hence from these tests it was further confirmed that the isolated strains belonged to the genus *Escherichia coli*. For molecular characterization of the *E.coli* strains, genomic DNA was isolated from 10 *E.coli* sp. by Phenol-chloroform extraction method.

The PCR test for amplification of ndvB gene has demonstrated that the gene can be detected and differentiated across a wide spectrum of *E.coli.* The PCR protocol described in the present study is inexpensive, accurate and rapid in detection of the ndvB genes in *E.coli* isolated from biofilm samples. Successful treatment of biofilm-associated infections is hampered due to high levels of resistance of biofilm-growing pathogens to antibiotics. It is difficult to completely kill microbial cells in a biofilm (especially those in the centre) by classical antibiotic treatment strategies, such as antibiotic prophylaxis, early aggressive antibiotic therapy and chronic suppressive antibiotic therapy.

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