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Biochemical Detection of N-Acyl Homoserine Lactone from Biofilm-Forming Uropathogenic *Escherichia coli* Isolated from Urinary Tract Infection Samples

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Abstract

Background: N-Acyl homoserine lactone (AHL) is found to be the main component of quorum sensing (QS) in Gram-negative bacteria and plays an important role in biofilm formation. Little information is available regarding the role of AHL in biofilm formation in *Escherichia coli (E. coli)*. The purpose of this investigation was to biochemically detect and characterize AHL activity in biofilm-forming uropathogenic *E. coli* (UPEC) isolated from urine samples of the patients with urinary tract infections (UTIs) in Kerman, Iran.

Methods: Thirty-five UPEC isolates were obtained from urine samples of the patients with UTIs referred to the Afzalipoor hospital. The isolates were identified by biochemical tests. Biofilm analyses of all the isolates were performed using the microtiter plate method at OD 490nm. N-Acyl homoserine lactone was separated from cell mass supernatants by liquid-liquid extraction (LLE) and analyzed by a colorimetric method. N-Acyl homoserine lactone functional groups were identified by Fourier Transform-Infrared Spectroscopy (FT-IR).

Results: The biofilm formation assay identified 10 (28.57%) isolates with strong, 16 (45.71%) with moderate, and 9 (25.71%) with weak biofilm activities. The UPEC isolates with strong and weak biofilm activities were subjected to AHL analyses. It was found that isolates with the highest AHL activities also exhibited strong adherence to microplate wells ($P \leq 0.05$). Two *E. coli* isolates with the highest AHL activities were selected for FT-IR spectroscopy. Peaks at 1764.33, 1377.99, and 1242.90 cm^{-1} correspond to the C=O bond of the lactone ring, and the N=H and C-O bonds of the acyl chain, respectively.

Conclusion: We found that many UPEC isolates exhibited strong biofilm formation. The control of this property by AHL may contribute to the pathogenesis of the organism in UTI's.

Keywords: Biofilm, FT-IR, N-acylhomoserine lactone, Uropathogenic *Escherichia coli*

Introduction

Many Gram-negative bacteria synthesize acyl-homoserine lactone (acyl-HSL) signal molecules that serve in a cell-to-cell communication system termed quorum sensing (QS). Quorum sensing regulates population density control of gene expression, biofilm formation, twitching motility, and virulence factor expression (1, 2). As the cells grow, members of a bacterial strain can communicate with each other by these diffusible QS autoinducers. Acyl-homoserine lactone, reported in more than 50 different bacterial species, consists of a lactone ring covalently linked to carbon acyl side chains through an amide bond (3, 4, 5). Acyl-homoserine lactones are lipid derivatives that differ from one another by the substituents and lengths of their acyl side chains (6), which can contain from 4 to 18 carbons.

Most N-acylated derivatives of L-homoserine lactone, such as acyl-HSLs, can differ with respect to the length, nature of the substitution at the 3-carbon position, and presence or absence of one or more unsaturation(s) within the acyl side-chain (4). Fig. 1 represents the chemical structure of an N-acyl

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homoserine lactone. R is a functional group that can be substituted by various fatty acid chains; for example, Pseudomonas aeruginosa (P. aeruginosa) relies on QS via production of the lactones N-butanoyl-l-homoserine (C4-HSL) and N-(3-oxododecanoyl)-l-HSL (3-oxo-C12-HSL) to regulate swarming, toxin and protease production, and proper biofilm formation. The absence of one or more components of the QS system can significantly reduce the pathogen's virulence. Little information is available regarding the relation of AHL and biofilm formation in UPEC strains (7).

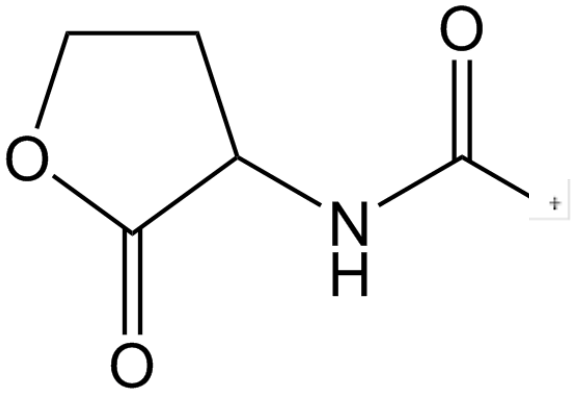

Fig. 1. General chemical structure of an N-acyl homoserine lactone. †= Fatty acid side chain.

Several studies have also suggested that biofilm formation is important for the development or persistence of some *E. coli*- associated infections. Intracellular biofilms, for example, may be important for the development of symptomatic urinary tract infections (UTIs) (8). A study by Ferrières et al., (9) found a strong bias for biofilm formation by asymptomatic bacteriuria strains. Not only did strains produce significantly more biofilm than UPEC strains, but they were also able to out-compete UPEC, as well as uropathogenic strains of Klebsiella spp., during biofilm formation.

The purpose of this investigation was to examine and characterize AHL activity in biofilm-forming UPEC isolated from UTI samples in Kerman, Iran.

Materials and Methods

Bacterial source

Thirty-five bacterial strains were recovered from samples obtained from patients with UTI's who were referred to the Afzalipoor university hospital in Kerman, Iran. Urine samples were collected midstream in clean, sterile 10 mL urinary containers, centrifuged at 5000 rpm (Sigma-1-15PK) for 5 min, and 0.1 mL of the lower portion of the pellet was inoculated into 5 ml of sterile Stuart transport medium (STM) (Merck, Germany). The samples were then transferred to the Department of Microbiology laboratory, Kerman University of Medical Sciences, for further analyses within 24 hours of sampling. Each patient's age and sex were recorded.

The samples were subjected to direct microscopic counts (bacterial growth $\geq 10^5$ colony-forming units (CFU)/mL), and the presence of leukocytes in the urine. The isolates were primarily inoculated on MacConkey and Eosin Methylene Blue (EMB) agars (Merck, Germany), and incubated at 37 ºC for 24 hr. The well-isolated colonies were identified by IMVIC tests, the Triple Sugar Iron (TSI) test, motility, Gram staining, and the ability to ferment various sugars. The identified isolates were mixed with 40% glycerol in DNase-free True North TM Cryogenic Vials (TNC) containing 1 mL of sterile Tryptic Soy Broth (TSB) (BioMérieux, Marcy l'Etoile, France) and stored at -70 °C.

Primary attachment assay

The initial attachment to surfaces is an important step in biofilm formation, as well as in the subsequent pathogenesis of biofilm-associated infections; therefore, we tested the ability of the strains to attach to polystyrene. Briefly, overnight cultures were adjusted to an optical density at 600 nm (OD600) of 0.5 (1×10^8 CFU/ml), diluted to 10^3 CFU/ml, and 100 µl aliquots of the suspensions were added to microtiter plate wells. After 30 min of incubation at 37 °C, the plates were gently rinsed three times with sterile 0.1M phosphate-buffered saline (PBS) and then covered with 150 µl of TSB. Primary attachment was expressed as the mean percentage of CFU (±SD) remaining on the microtiter plate relative to the initial inoculums.

Biofilm formation assay under static condition

Formation of the biofilm in each UPEC isolate was quantified by the microtiter plate method as described previously (10) with some modifications. Briefly, one loopful from each *E. coli* colony was inoculated into 2 mL of sterile TSB medium containing 1% W/V glucose to optimize biofilm production. The OD600 was then adjusted to one to achieve 0.5 McFarland standard (1.5×10^8 CFU/mL) followed by further dilution of the prepared bacterial suspension to achieve ~10^6 CFU/mL. One hundred µL of each prepared inoculums were added to wells of 96-well polystyrene flat-bottom tissue culture microplates. Similarly, 100 µL from each of the standardized cell suspensions were transferred to the wells of round-bottom 96-well micro-titer plates (MTP). One hundred µL of uninoculated medium were used as negative controls. Bacteria were allowed to adhere and grow without agitation for 24 hr at 37 °C. After incubation, media containing non-adherent cells were aseptically aspirated, washed, and replaced with 10 µl of sterile PBS (pH 7.2). Both cell viability and biofilm biomass were determined as described previously (10). Consequently, 150 µl of methanol were added to each well and plates were incubated at room temperature for 20 min. The methanol was then removed and replaced with 200 µl of 1% W/V crystal violet solution. The wells containing the biofilm matrix were washed gently with sterile deionized water and the plates were kept at room temperature until dried. Thereafter, 200 µl of 33% V/V glacial acetic acid were added to each well and the OD was measured at 490 nm using a Synergy 2 multi-mode microplate reader (BioTek, USA). The isolates were classified as strong, moderate, weak, or no biofilm formation based on a formula introduced by Stepanović et al. (10). Simultaneously, CFU/mL of each isolate in the wells were determined and subtracted from CFU/mL in control samples. All experiments were performed in
triplicate and results were expressed as means ± SD's.

Detection of AHL by colorimetric method

The AHL activities of the *E. coli* isolates were detected by a colorimetric method as described previously (11). Briefly, the bacterial isolates were grown overnight in 5 mL of sterile Muller-Hinton broth (Merck, Germany) at 37 ºC; then, 1.5 ml of the overnight cultures were aseptically transferred into sterile centrifuge tubes (Eppendorf, Germany) and centrifuged at 10,000 rpm for 15 min. The cell pellets were discarded and the procedure was repeated three times. The supernatants were filtered through 0.2 µm membrane filters (Sartorius, Germany) to remove the cell debris. Then, filtrate were mixed with of ethyl acetate and shaken for 10 min. The mixture was allowed to stand for five min in a separating funnel to form upper (organic) and lower (aqueous) immiscible. The upper part was collected in sterile tubes and the lower portion was extracted two more times as described above.

The upper portion from each sample were pooled and dried in an oven at 40 ºC. 40 µL of each were prepared by liquid-liquid extraction (LLE) (12) and inoculated into wells of 96-well polystyrene flat-bottom tissue culture microplates supplemented with and 50 µL of a 1:1 mixture of hydroxyl amine (2 M): NaOH (3.5 M) was aliquoted and mixed with the sample. Subsequently, the same amount of 1:1 mixture of ferric chloride (10% in 4 M HCl):95% ethanol was added The OD was measured at 520 nm. The AHL extracts were concentrated in an oven at 40 ºC overnight and stored at −20 °C for further analyses. A dark brown color was shown from all of the samples containing lactone compounds, but in some cases repetitive pippeting of the mixtures changed it to a yellow color, depending on the concentrations of lactones. Under alkaline conditions AHLs are rapidly inactivated by pH-dependent lactonolysis, in which the homoserine lactone ring is hydrolyzed to open ring form corresponding to N-acylhomoserine. This reaction can be reversed by acidification; therefore, we monitored pH throughout the experiment to verify that an acidic pH was maintained.

Determination of AHL functional groups

N-Acyl homoserine lactone functional groups were identified by FT-IR as described (13). Briefly, a drop of the AHL extract by was placed on a KBr plate, a second plate was placed on top, and the samples were subjected to IR (Brauker Tensor 70 FT-IR Spector-photometer).

Statistical analyses

Pearson's χ^2 and Fisher's exact tests were performed to compare the number of isolates producing AHL. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA). A P-value≤0.05 was considered statistically significant for two-tailed tests.

Results

The biofilm formation assay identified 10 (28.57%) strong, 16 (45.71%) moderate, and nine (25.71%) weak biofilm-producing isolates (Fig. 2). The 10 UPEC isolates with strong biofilm activity were subjected to AHL quantification assay as shown in Fig. 3. It was found that isolate 28 had the highest, and isolate 33 the lowest, AHL activities at OD 520 nm (1.552 and 0.689, respectively) (Fig. 3). Two isolates (28 and 11) that demonstrated the highest AHL activities were selected for analyses of their functional groups by FT-IR spectroscopy (Fig. 4). Peaks at 1764.33, 1377.99, and 1242.90 cm^{-1} correspond to the C=O bond of the lactone ring, the N=H bond, and the C-O bond, respectively. These results support the AHL data and confirm the presence of a lactone ring in the AHL produced by UPEC strains. Biofilm formation was observed for most isolates.

Discussion

It is well known that biofilm-forming ability is mainly due to the amount of AHL produced by microorganisms, as occurs in the QS system (7). Here we observed that the two UPEC strains with the highest AHL activities exhibited strong biofilm formation.

Van-Houdt et al. (15) reported that *E. coli* has the capacity to alter its pattern of gene expression and phonotypical properties in response to AHLs by means of the AHL-responsive transcriptional regulator SdiA. Similarly, in *E. coli*, cell division may be partially regulated by AI-2-mediated QS (13). *E. coli* and *Salmonella enterica* do not produce AHL signals commonly found in other Gram-negative bacteria; however, they have a receptor that detects AHLs from other bacteria and change their gene expression in accordance with the presence of other populations of Gram-negative bacteria (16). Several recent studies have addressed the regulatory effect of cell-signaling mechanisms mediated by AHL on the process of biofilm formation in various Gram-negative bacteria, including *P. aeruginosa* (17), *Serratia liquefaciens* (18), and *Burkholderia cepacia* (19). Although there is no direct evidence for a mechanistic relationship between QS and biofilm formation in *E. coli*, in the present study we detected biofilm-forming UPEC capable of producing AHL at relatively high levels. Further research is needed to understand the molecular genetics of this compound in *E. coli*.

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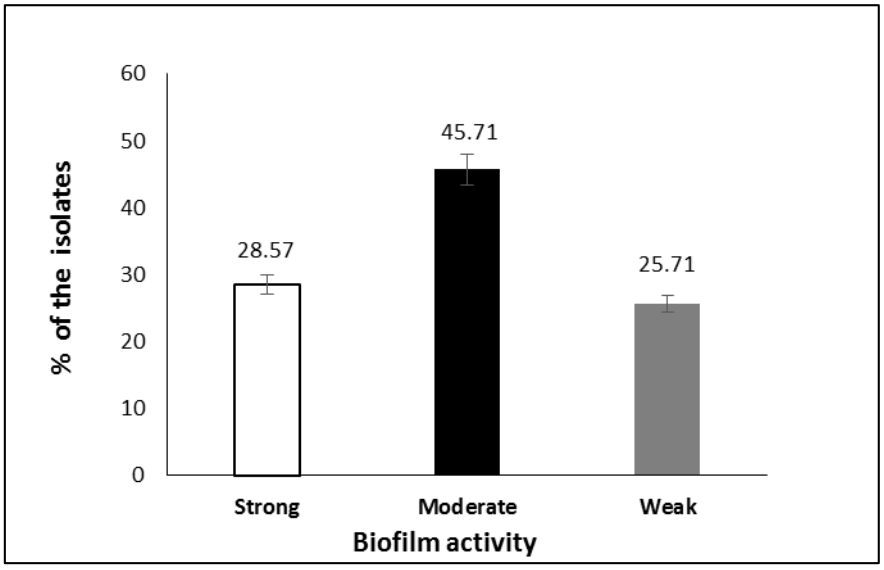

Fig. 2. Biofilm activity of 35 strains of uropathogenic *E. coli* isolated in this study. The amount of biofilm remaining was determined by the absorbance of the crystal violet dye. Error bars represent the standard deviation from the mean of three observations. A standard culture of *P. aeroginosa* PAO1 was used as positive control.

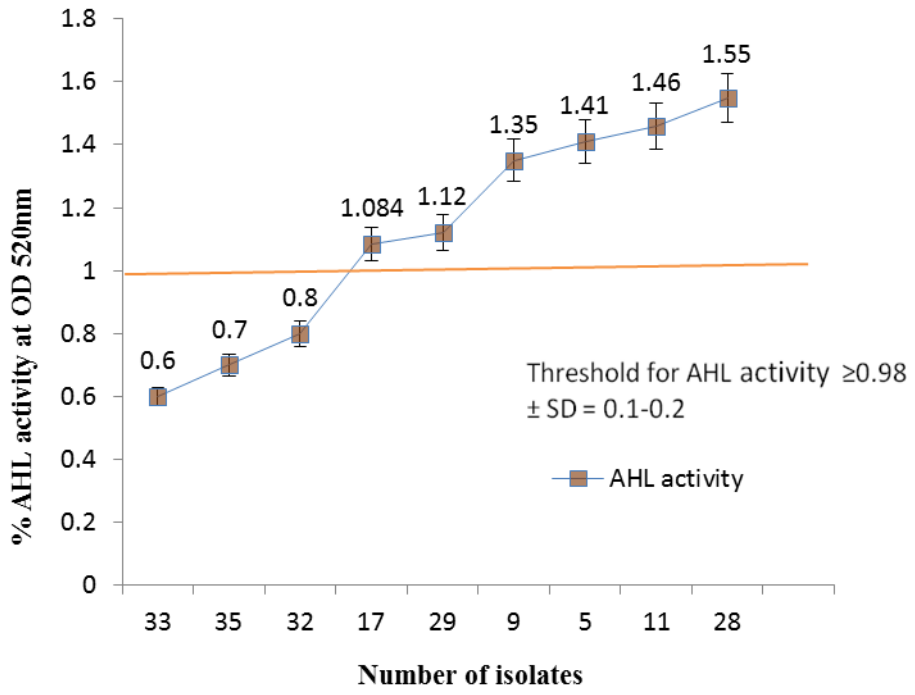

Fig. 3. AHL- activity by uropathogenic *E. coli* exhibiting strong biofilm. The results below threshold indicate weak or no AHL activity. The results were means of three replicate experiments.

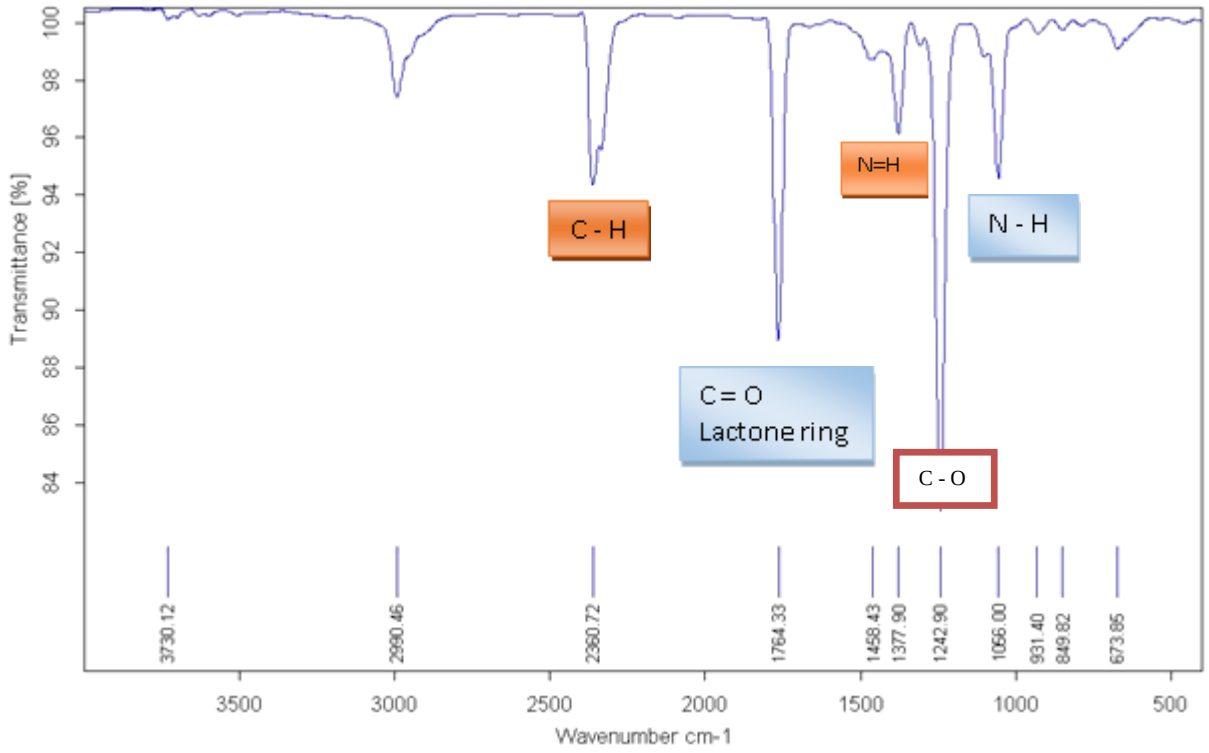

Fig. 4. FT-IR spectra of AHL functional groups produced by uropathogenic *E. coli*. The AHL was extract from organism by LLE- methods as described in the text. The pure compound was then subjected to FT-IR spectroscopy. The lactone ring was shown at 1764.69cm-1 wave number.

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