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The effect of D and L- amino Acids on Biofilm Formation in Different Microorganisms

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Abstract

The D-enantiomers of amino acids have been thought to have relatively insignificant function in biological processes like, D-amino acids are sometimes found in proteins that are not synthesized by ribosomes. While L-amino acids clearly permanent in nature, D-amino acids have previously inapprehensible regulatory roles in the bacterial kingdom, any diverse of bacterial phyla made from these D-amino acids regulate cell wall remodeling in stationary phase and cause biofilm dispersal in aging bacterial communities. Clarification the mechanism by which D-amino acids given cell wall reorganization and biofilm disassembly will undoubtedly discover new paradigms for understanding how extra cytoplasmic processes are regulated as well as lead to development of novel therapeutic. Results of this study evaluated that 50 and 100mM of D-glycine have inhibitory effect on *Klebsiella pneumonia*, and *Staphylococcous aeurues* biofilm formation. Also50 and 100mM of D-aspartic acid have the same inhibitory effect on *Escherichia coli*, and *Staphylococcous aureus* biofilm formation. The mix of 100Mmof both D-glycine and aspartic acid have more effective inhibitory activity on *Escherichia coli*, and *Staphylococous auerues* biofilm formation than when it used alone. While the use of L-serine, L-isoleucine and L-tyrosine have no inhibition activity on biofilm formation of *Klebsiella pneumonia*, *Staphylococcous aurues* and *Escherichia coli*.

Keywords: biofilm inhibition, D-amino acids.

تأثير الأحماض الامينية من نوع L و D على تكوين الغشاء الحياتي في احياء مجهرية مختلفة

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الخلاصة

المتماثلين صورياً من نوع D للأحماض الأمينية كان من المعتقد إنه يملك وظيفة ثانوية نسبياً في العمليات البايولوجية. مثلاً ، بعض الأحيان الأحماض الأمينية من نوع D تتواجد في البروتينات التي لا تصنع بوساطة الرايبوسوم. بينما الأحماض الأمينية من نوع L غالباً وبشكل واضح تتواجد في الطبيعة، الاحماض الامينية من نوع D لها دور تنظيمي غير مقدر سابقاً في المملكة البكتيرية، أي تنوع في العوائل البكتيرية جعل الأحماض الأمينية من نوع D تنظم إعادة عرض الجدار الخلوي خلال طور الثبات وتسبب تشتيت الغشاء الحياتي في المجاميع البكتيرية الهرمة. توضيح الميكانيكية التي بواسطتها الاحماض الامينية من نوع D تُعيد تنظيم الجدار الخلوي وتفكيك الغشاء الحياتي، مما لاشك فيه سوف يكشف نموذج جديد كيف تنظم العمليات خارج الخلوية وكذلك يؤدي لتطوير علاج جديد. تهدف الدراسة الى معرفة تاثير الاحماض الامينية من نوع D و L على تكوين غشاء حياتي في عزلات بكتيرية مختلفة ، اوضحت نتائج الدراسة بان استخدام50أو100ملي مولاري من D-gycine له تاثيرات تثبيطية على تكوين الغشاء الحياتيفي *Klebsiella Klebsiella pneumonia* و*Staphylococcus aureus* وكذلك استخدام 50 أو100 ملي مولاري من D-aspartic

acid له نفس التاثيرات التثبيطية على تكوين الغشاء الحياتي في *Escherichia coli* و *Stapylococcus aureus*. كما ان دمج 100 ملي مولاري من كلا الحامضين الامينيين له فعالية تثبيطية اكثرعلى تكوين الغشاء الحياتي في*Staphylococcus aureus* من استخدامه وهو مفرد. لكن لوحظ ان استخدام L- isoleuine و L-Serine وL-tyrosinلم يكن له اي فعالية تثبيطية على تكوين الغشاء الحياتي في عزلات *Klebsiella pneumonia* و*Staphylococcous aeurues* و*Escherichia coli*.

Introduction

Biofilm are communities of microorganisms in a matrix that joints them together and to livening inert substrates. Many bacteria can produce agents that prevent biofilm formation. In one recent example, D-tyrosine, D-phenylalanine and D-proline, as well as a mixture of D-amino acids, were shown to be inhibitory effective in*Staphylococcous aeurues* biofilm formation [1-5]. One mechanism could be the advanced D-amino acids disassemble biofilm was incorporation of D-amino acids in peptide side chain of peptidoglycan place of the terminal D-alanine [3,4]. Additionally, biofilms that were treated with D-amino acids were found to have less surface proteins when compared to biofilm treated with L-amino acids by confocal microscopy [2]. The anti-biofilm characteristics of D-amino acids are not restricted to *Staphylococcous aeurues*, as the authors found that D-amino acids also prevented biofilm formation in other bacteria, such as Bacillus subtilis and Escherichia coli, considering D-amino acids are produced in late biofilm culture by B. subtilis, there may be some, general tell us nature to this biofilm dispersal across bacterial species [6].

In this work attempted to know if there is an important role of D and L amino acids on biofilms formation in *Klebsiella pneumonia*, *Staphylococcous aeurues* and *Escherichia coli* isolates.

Material and Method

Selection of the Isolates:

The bacterial strains used in this study were include 10 isolates from each *Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumonia*, isolated from 30 urine, blood, stool, sputum, burn, wound, ear water, sewage, soil specimens. Those isolates showed increase resistance to commonly available antibiotics by using Kirby- Baur disc diffusion method. And identified by standard microbiological procedures (Gram staining, colonial morphology, catalyses test, cytochrome oxidase reaction, motility, biochemical tests) which carried out depending on Berge's manual of systematic Bacteriology [7], also by analytical profile index (API) 20 E system and vitek 2 system [8].

Determination of Minimum Inhibitory Concentration (MIC):

D-glycine, D-aspartic acid, L- serine, L-isoleucine, L- tyrosine were prepared to determine the MIC for plank tonic cells. A stock solution of 1 M of each amino acid was prepared in distilled water. The stock solutions were filter-sterilized by passage through 0.45 ɱm membranes (Billerica, MA. USA). These were prepared to achieve different molarities of each amino acid, starting with 100mM and serial dilution was done with the medium to the end point concentrations. MIC test were conducted in 96 flat bottom microtiter plates (TPP, Switzerland). Each test well was filled with 100 ml nutrient broth. A 100 µl of the stock solution was added to the first test well and mixed, then a series of dilutions was prepared across the plate after that 10 µl of liquid of the microorganism (*Staphylococcus aureus, Escherichia coli,* and *Klebsiella pneumonia*), was used to inoculate each microtiter plate well to achieve a final inoculums size of $4x10^5$ CFU/ml well with overnight culture. Nutrient broth and bacterial inoculums but without amino acid treatment were assigned as positive growth controls, whereas negative controls were D-amino acid treated wells but without inoculums. All control wells were prepared and incubated under the same experimented conditions. Plates were incubated for 24hr at 37Cº. The wells were examined for microbial growth by naked eye. The MIC value was described as the lowest D –amino acid concentration that inhibited about 80% of microbial growth, relative to the negative and positive controls, microbial growth in the test wells was detected as turbid. MIC determination was carried out in triplicate [9].

Biofilm Formation Assays by using Tissue Culture Plate (TCP) Method:

This quantitative test described by Hassan *et al* (2011) [10], considered the gold standard method for biofilm detection. Organisms isolated from fresh agar plates were inoculated in 10 ml of trypticase soy broth with 1% glucose w/v. Broths were incubated at 37Cº for 24 hours. The culture were then diluted 1:100 with fresh medium and inoculated individual wells of sterile 96 well- flat bottom polystyrene tissue culture plate. Negative control wells contained inoculated sterile broth. The plates

were incubated at 37Cº for 24hrs.After incubation content of each well were removed by gentle tapping. The wells were washed with sterile distilled water once. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were stained by (0.1%) w/v crystal violet. Excess stain was removed by using distilled water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader (model 680, Biorad, UK) at wavelength 630 nm, and the interpretation of the results was conducted as shown in Table-1. The experiment was performed in triplicate and repeated three time [11, 12].

Table 1- Interpretation of Biofilm production

Average OD value	Biofilm production
≤ OD /ODc < ~ ≤ 2x ODc	Non / weak
2x ODc < ~ ≤ 4 x ODc	Moderate
> 4x ODc	Strong

- Optical density cut- off value (ODc) = average OD of negative control + 3x standard devation (SD) of negative control [10]

The Effect of D-amino Acids on Biofilm Formation

Biofilm formation assays were performed using 96_ well microtiter plate, based on the protocol by Goh, S. *et al* (2013) [13], with minor modifications. Briefly *Escherichia coli* ,*Staphylococcous aeurues* and *Klebsiella pneumonia* were cultured in TS Broth overnight and the resulting culture was diluted to 1:100 (TSB + 1% w/v glucose) [14]. Each well of microtiter plate was loaded with 100 ml of medium and 100 µl of 50 or 100 mM of each amino acid, except a control well without any amino acid. Each concentration for every amino acid tested was assayed triplicate. The plate then incubated at 37Cº for 24 hrs. The plank tonic bacteria were removed by shaking the dish over a waste tray filled with sterile distilled water. Subsequently 0.1% w/v crystal violet solution was added to each well and the plate was left to stain for 10 min at room temperature. Next the crystal violet solution was removed by submerging the plate in a water tray. The plate was then inverted and topped on paper towels to remove excess liquid and left to air dry. The stained wells were then treated with 95% v/v ethanol for 10 min at room temperature to solubilize the dye. The bacterial suspension in each well was mixed well and its optical density was measured in a micro plate reader at 630nm.Also the effect of mixing 100mM of D-Glycine with D-aspartic acid were prepared by loaded 50µl of each amino acid with 100 µl of medium and the other steps were the same.

Statistical Analysis

All the assays were compared using T-test analysis of variance. Differences were considered significant when $P< 0.05$ [15].

Result and Discussion

Among 30 isolates of TCP; 6 were produced strong biofilm, 21 were moderate and 3 were weak or non –biofilm producers as shown in Table-2:

Table 2- Screening of the Isolate for Biofilm Formation by Tissue culture plate

	Biofilm Formation	TCM n(%)
No .of isolates(30)	High	6(20%)
	Moderate	21(70%)
	Weak /None	3(10%)

The number of isolates produced biofilm formation was 27 (90%) and none or weak biofilm were 3 (10%). Hassan *et al* (2011) [10], also showed that out of 110 isolates tested, the number of biofilm producers were70(64.7%)and non or weak biofilm producers were 40 (36.3%). Regional data from India also showed that out of 152 isolates tested, the number of biofilm producers was 53.9% and non-biofilm producers were 46% [11]. In recent study the majority of organisms associated with biofilm production were *klebsiella pneumonia* (37.03%) fallowed by *Escherichia coli* (33.3%), and *Staphylococcus auereus*(29.62%). The results interverse with the result of which reported that the Hassan *et al* (2011) [10], were reported that the majority of organisms associated with biofilm production were *Staphylococcus epidermidis* (37.1%) followed by *Escherichia coli* (27.1%), *klebseilla pneumonia*(15.7%) *Staphylococcus aureus* (11.4%), *Enterococcus faecalis*(4.2%)and *Pseudomonas*

aueroginosa (4.2%). Biofilm producing bacteria were isolate from urine (22.2%) followed by blood (18.5%), wound (14.8%), sewage (11.1%), all the others ear, soil, sputum, stool, burn were (7.4%).This results similar to Hassan *et al* (2011) [10] results, and Donlonand Costerton results (2002) [16], reported that the association of biofilm producing bacteria with urinary tract infection. As shown in Table-3.

Table 3- The correlation between Biofilm Production and Type of Isolates

Organism	Biofilm Production	Type of Isolates
1 *Escherichia coli* isolate	moderate	urine
1 Klebsiella pneumonia isolate	strong	Urine
1 Klebsiella pneumonia isolate	moderate	Urine
2 Staphylococcus aureus isolate	moderate	Urine
2 Escherichia coli isolate	moderate	Blood
3 Staphylococcus aureus isolates	moderate	Blood
3 Escherichia colli isolates	moderate	Sewage
2 Klebsiella pneumonia isolate	strong	Wound
2 Staphylococcus aureus isolate	moderate	Wound
2 Staphylococcus aureus isolate	moderate	Ear
2 Klebsiella pneumonia isolate	moderate	Soil
1 Klebsiella pneumonia isolate	moderate	Sputum
1 Klebsiella pneumonia isolate	strong	Sputum
2 Escherichia coli isolate	moderate	Stool
2 Klebsiella pneumonia isolate	strong	Burn

The study determined the minimum concentration needed to prevent plank tonic cells to produce biofilm, individual D-amino acids equal in their activity, for 50mM D-glycine and D-aspartic acid. While L-tyrosin, L-isoleucine and L-serine have no activity to prevent plank tonic cells as shown in Table-4.

Table 4- MIC value of D and L-amino acids of different microorganisms

Strains	MIC (mM) D,L-amino asid				
	D-glycine	D-aspartic asid	L-isoleucine	L-tyrosin	L-serine
Staphylococcus aureus	50	50	-	-	-
Escherichia colli	50	50	-	-	-
Klebsiella pneumonia	50	50	-	-	-

The ability of 30 isolate from each *Eschericia.coli*, *Klebsiella pneumonia*, and *Staphylococcous aeureus* to produce biofilm were revaluated by using pre-sterilized 96 well polystreren microtiter plates and then read absorbance were at 630 nm in an ELISA reader for determination of biofilm formation degree studied strains that adhered on the surface of the microtiter well , absorbance values were represented the degree of the biofilm thickness that formed by the studied strains on the surface of the microtiter well all 30 isolate assay for production of biofilm.The difference in biofilm thickness result from different reasons such as differences in isolates capacity to form biofilm,perhaps the primary number of cells that succeeded in adherence and the differences of quality and quantity of auto inducers (Quorum sensing signaling molecules) that produced from each isolate andplay an essential as well as important role in biofilm formation [17],as reported in Awad, (2012) [18],which found that (52%) of the tested isolates were high producers while (28%) Isolates were good producers and (20%) were poor produces, moreover *Klebsiella pneumonia* ($2K_8$) which isolated from sputum produced the thickest biofilm with O.D0.344. Biofilm formation in 30 isolates was measured spectrophotometrically following incubation in the presence of D-glycine, D-aspartic acid in various molarities concentration 50 and 100 mM. With increasing molarities concentration of D-amino acid the optical density also decrease corresponding until the concentration of 100mM.

We observed marked significant differences (P value ≥0.05) in biofilm formation with increase molarities concentration of D-glycine, aspartic acid in both *klebseilla pneumonia* and *Staphylococcus aureus* isolates also with the use of mixture of 100Mm of D-glycine, aspartic acid in both *Escherichia coli* and *Staphylococcus aureus* isolates as shown in Table -5.

Table 5- The significant differences of D-amino acid on different Microorganism

Escherichia colli	*Klebsiella pneumonia*	*Staphylococcus aureus*
D-glycine 50 mM		
3/10	5/10	4/10
D-glycine 100 mM		
3/10	10/10	9/10
D-aspartic acid 50 mM		
4/10	5/10	7/10
D-aspartic acid 100 mM		
9/10	4/10	9/10
Mix (100 mM D-glycine +100 mM D-aspartic acid)		
9/10	3/10	10/10

In contrast, L-amino acid alone or in mixture neither inhibited biofilm formation nor distributed existing biofilm. The inhibition activity was mentioned in the article initially reporting the effect of D-amino acids on biofilm growth [4]. In other report observed that glycine showed an inhibitory effect on biofilm formation and the extent of inhibition was concentration-dependent [19]. In regard to results of this study a significant decrease in biofilm growth was observed at 4% concentration in *Escherichia coli* [13]. D-aspartic acid which inhibited biofilm formation on tissue culture plates similar to Hang.Y.M et al(2015) [20], indicates that the high concentration above 10mM inhibited the growth of *staphylococcus aureus* plank tonic cells. The decrease cellular metabolism activity might be the reason for producing less protein and DNA in the matrix of the biofilm formed in the presence of aspartic acid. However varied inhibition efficacies of aspartic acid were observed for biofilm formed by clinical Staphylococcus aureus isolates. There might be mechanisms other than decreasing the metabolic activity e.g. the biofilm phenotypes affecting the biofilm formation in the presence of aspartic acid. It is believe that the mode of action of D-amino acid in biofilm formation is by prevented initial attachment which is the primary steps of biofilm formation in bacteria by reducing extracellular polysaccharides and protein production in early growth stage [3].

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