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The Efficiency of Certain Amino Acids in Regulating *chABCI* Gene Expression in *Proteus mirabilis*

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Abstract

Many previous studies have demonstrated the therapeutic importance of chondroitinase ABC encoded by the *chABCI* gene. This study, however, accomplished to determine some amino acids effects on the *chABCI* gene in *Proteus mirabilis* to regulate chondroitinase production. A total of 57 (96.61%) isolates of *Proteus mirabilis* and two (3.39%) isolates of *Proteus vulgaris* were obtained from clinical specimens. The impact of amino acids on bacterial growth was assessed by gauging the turbidity of the nutrient broth using a spectrophotometer. Two isolates of *P. mirabilis* were treated with amino acids and their effect on the gene expression of the *chABCI* gene was studied using a real-time polymerase chain reaction. L-tyrosine and L-serine significantly increased bacterial inhibition ($p < 0.05$), while L-arginine, L-glutamine, L-proline, L-cysteine and glycine didn't significantly affect *P. mirabilis* growth ($p > 0.05$). Also, gene expression increased significantly ($p < 0.05$) for *chABCI* with varying degrees when treated with these amino acids in addition to L-serine. Whereas L-tyrosine had a significant effect in reducing gene expression ($p < 0.0001$). It can, therefore, be concluded that amino acids significantly affect the gene expression of *chABCI*, either by increasing or inhibiting it.

Keywords: Chondroitinase ABC I, *chABCI*, Amino acids, *Proteus mirabilis*, Gene expression

كفاءة بعض الأحماض الأمينية في تنظيم التعبير الجيني لـ *chABCI* في *Proteus mirabilis*

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الخلاصة

أظهرت العديد من الدراسات السابقة الأهمية العلاجية لـ Chondroitinase ABC المشفر بواسطة الجين *chABCI*. لذلك أنجزت هذه الدراسة لتحديد تأثير بعض الأحماض الأمينية على جين *chABCI* في *Proteus mirabilis* لتنظيم إنتاج إنزيم الChondroitinase. تم الحصول على 57 عزلة (96.61٪) من *Proteus mirabilis* وعزلتين (3.39٪) *Proteus vulgaris* من العينات المعزولة سريريا. وتم تقييم تأثير بعض الأحماض الأمينية على نمو هذه البكتيريا عن طريق قياس التعكر في المرق المغذي باستخدام مقياس الطيف الضوئي. عوملت عزلتان من *P. mirabilis* بالأحماض الأمينية ودُرس تأثيرها على التعبير الجيني لجين *chABCI* باستخدام تفاعل البلمرة المتسلسل اللحظي. زاد كل من L–tyrosine و L–serine بشكل معنوي ($p <0.05$) ، بينما لم يؤثر L–arginine وL–glutamine فضلا عن L–proline و من تثبيط البكتيريا

L–cysteine و الحامض الاميني glycine بشكل معنوي ($p < 0.05$) على نمو *P. mirabilis* . كذلك زاد التعبير الجيني معنويا ($p < 0.05$) لـ *chABCI* وبدرجات متفاوته عند معاملته بنفس هذه الاحماض الامينية بالاضافه الىL–serine ، بينما كان لـ L–tyrosine تأثير معنوي في تقليل التعبير الجيني ($p < 0.0001$) لهذا الجين. يمكن الاستنتاج أن الأحماض الأمينية تؤثر بشكل معنوي على التعبير الجيني لـ *chABCI* ، إما عن طريق زيادة التعبير أو تثبيطه.

Introduction

Chondroitinase ABC (ChABC) is an enzyme produced by *Proteus vulgaris* [1]. This enzyme is active on the chondroitin sulfate and dermatan sulfate, due to having a lyase that is responsible for breaking down the side chains of the proteoglycan molecules [2]. This is what inspired many researchers to investigate its potential as a treatment despite being a virulence factor [3]. Proteoglycans are involved in matrix organization and structure, cell proliferation, migration, and cell surface signal transmission [4]. According to many studies, chondroitin sulfate proteoglycans (CSPGs) are generally blamed for impeding tissue regeneration in several diseases, including cancer and neurological disorders such as traumatic brain injury, neurodegenerative, spinal cord injury, and are primarily suspected of impeding tissue repair [5, 6]. In such a situation, the therapeutic enzyme chondroitinase ABC, which is principally responsible for breaking down the side chains of the proteoglycan chondroitin sulfate molecule, has shown tremendous promise for treating these disorders in multiple preclinical investigations [7, 8]. However, due to their limited expression and low enzyme activity, in addition to expensive complex extraction methods, the majority of Ch ABCs still fall short of industrial requirements where their future applications on a larger scale are hindered [8, 9].

Bacteria react quickly to changes in the environment and within the cell. Controlling the expression of genes at the level of protein creation is a crucial method for quickly detecting and adjusting to changes in environmental circumstances [10]. Cells use stimuli to adjust the rate of translation and cellular protein concentrations at each of the three main stages of translation: initiation, elongation and termination. For instance, variations in the cell nutrient concentrations may trigger translational reactions involving systems that significantly alter the physiology of the cell. Additionally, by controlling gene expression, the cell can precisely control the level of specific protein products [11].

Numerous phenotypic research has examined how amino acids affect microorganism growth and metabolism. However, the precise mechanism of amino acids' action in gene regulation is not well understood. Scientists had the idea of increasing the expression of certain metabolic pathway enzymes using strong promoters [12]. This study focused on the use of some amino acids to find out their effect on the gene expression of *chABCI* which is responsible for the production of the chondroitinase ABC enzyme, to study the possibility of increasing its production in the laboratory for its therapeutic importance.

Material and Method

Bacteria Isolation and Identification

A total of 460 specimens of urine (234), wounds (74), burns (63) and ear (89) swabs were collected from several hospitals in Baghdad, including Baghdad Teaching Hospital, Ghazi Hariri Hospital, Al-Yarmouk Teaching Hospital, and Al-Kadhimiya Teaching Hospital. The identification of *Proteus* species was conducted through various standard morphological and biochemical tests. Initially, the isolates were inoculated on MacConkey agar and blood agar plates to observe their growth characteristics. Further identification was supported by biochemical tests such as oxidase and catalase testing. Indole and urease production abilities were assessed, as well as the capacity to utilize citrate as a carbon source. Finally, the identification of the isolates was confirmed using the VITEK 2 system (bioMérieux) with the VITEK® 2 GN kit, following the manufacturer's instructions.

Preparation of Stock Solution

After determining the amount of amino acid powder and water needed to make the stock solution according to the Merck Index [13]. L-serine (Ser), L-glutamine (Gln), L-tyrosine (Tyr), L-arginine (Arg), L-proline (Pro), and Glycine (Gly) were prepared at a 100 mM concentration. L-cystine (Cys) was initially dissolved in 1 M HCL until it yielded a colorless, clear solution and then was diluted with distilled water to obtain 100 mM. All amino acids were obtained from Himedia (India).

Effect of Amino Acids on the Growth

To determine whether amino acids have any effect against *P. mirabilis*, concentrations of amino acids 100 mM were added into tubes containing nutrient broth and adjusted to pH 7 with NaOH and HCL solution. Additionally, a control set was generated in which the bacteria were grown in the same media without any exposure to amino acids. After that, all tests were incubated for 24 hours at 37°C. Following the incubation, the growth media turbidity was measured at 600 nm using a T60 UV-visible spectrophotometer [14]. In this part of the research, three replications were conducted to examine the growth.

Polymerase Chain Reaction Primer Design

Primers of *chABCI* and *16S rRNA* (Table 1) were designed by Geneious Prime software. The sequence of *Proteus vulgaris* strain ATCC 6896 (https://ncbi.nlm.nih.gov/nuccore/GQ996964) was used to design the primer of the *chABCI* gene. The figure in the appendix shows the binding site of the primer that was designed. The OligoAnalyzer tool from IDT and certain other different online tools were used to check the primers. The origin of each primer used in this research was Macrogen® (Korea).

Table 1: Primer used in this study

Primer's Name	Sequence	Product Size
chABCI	F 5'- CCAAAGTCATGGTGTCGCTCA-3' R 5'- TGTTCCGCTAAATCCACGCTC-3'	184 bp
16S rRNA	F 5'AGCGGGGAGGAAGGTGATAAA-3' R 5'-CTCGGGGCTTTCACATCTGAC -3'	177 bp

Detection of *chABCI* Gene

The total DNA was extracted following the manufacturer's instructions of Norgen's Bacterial DNA Isolation Kit (Canada). OneTaq (NEB®, England) master Mix was used when amplifying fragments of the gene by PCR to detect the gene of interest. The appropriate PCR conditions are listed in Table 2. The Sambrook and Russell standard method was followed to prepare horizontal agarose gel electrophoresis to migrate the PCR amplification product [15]. Ten µl of each DNA sample of the final reaction mixture and 25 bp -ladder (NEB®, England) were loaded to the wells of the 2% agarose gel after staining with RedSafe dye (Intron, South Korea). Finally, the amplified products were observed by gel documentation (Cleaver, U.K).

Table 2: PCR conditions for the *chABCI* gene that apply in the thermocycler

Cycle No.	Stage	Temperature	Time
1	Initial Denaturation	94°C	5 mins.
35x	Denaturation Annealing Extension	94°C 55°C 72°C	30 sec. 45 sec. 45 sec.
1	Final Extension	72°C	7 mins.

Growth Medium, and Cultural Conditions for Real Time-PCR

To prepare the medium, meat extract 2.3 g (Himedia), sodium chloride 1.5 g (Himedia), peptone 15 g (BDH, England), and chondroitin sulfate 1 g (NUTRABIO, USA) were dissolved in 1000 ml of distilled water, and the pH was adjusted to 8 [16]. Bacteria were grown in this medium for two days at 37°C for both the control group and those that had been treated with amino acids at 100mM concentration.

Real Time-PCR Assay

Total RNA was extracted and purified according to the TRIzol™ Reagent protocol (Invitrogen, USA). Later transcribed RNA reversely to complementary DNA (cDNA) to serve as a template using ProtoScript cDNA Synthesis Kit (NEB, UK). Then the cDNA of bacteria post-treatment was performed with 100 mM concentration of amino acids and the cDNA of the same bacteria without treatment as a control was included in the same run. Two PCR tubes were used for each sample, one tube for the *chABC1* gene and the other for *16S rRNA* (Housekeeping) as a control for analysis. Quantity detection was based on SyberGreen fluorescence power. The volume of the master mix and other components of the reaction are shown in Table 3. Following the recommended thermal cycling real-time PCR software was set up (Table 4)

Table :3 qRT-PCR reaction mixture

Component	Volume (µl)
Luna Universal qPCR Master Mix	10
cDNA	5
F-primer (10µM)	1
R-primer (10µM)	1
Nuclease-free Water	3
Total	20

Table :4 Set up of Real-Time PCR

Stage	Temperature	Time	Cycles
Initial Denaturation	95°C	60 sec.	1
Denaturation **Extension**	95°C 60°C	15 sec. 30 sec.	45
Melt Curve	60-95°C	40 min.	1

Analyzed RT-qPCR Data

Fold change values were calculated by following Livak's method for qPCR data analysis [17].

$$fold\ gene\ expression = 2^{\Lambda} - (\Delta\Delta Ct)$$

Statistical Analysis

Data was presented as mean ± SD values. The data were statistically analyzed by unpaired Student's t-test using SPSS version 21, software (San Diego, California, USA). *P*-values ≤ 0.05 were considered to be significant, and *p*-values > 0.05 stood for non-significant.

Results and Discussion

A total of 460 specimens were collected from people suffering from various diseases attending several hospitals in Baghdad. These specimens consisted of 234 urine, 63 burns, 74 wounds, and 89 ear swabs. The findings of the present study revealed a prevalence rate of 12.83% (59 isolates) for *Proteus* spp. Among the 59 isolates belonging to the *Proteus* species, the prevalence percentage of *P. mirabilis* was higher compared to *P. vulgaris*. Specifically, 57

isolates (96.61%) were identified as *P. mirabilis*, while only two isolates (3.39%) were identified as *P. vulgaris*, aligning with findings from other studies [18, 19].

Effect of Amino Acids on the Growth

It is well known that some amino acids inhibit bacterial growth. The turbidity of the growth media treated with glycine, L-glutamine, L-cystine, L-proline, L-arginine, and the growth media not treated showed identical outcomes, while the L-tyrosine growth media was not turbid compared to the control which indicated an increase in the bacterial inhibition rate. The OD of media treated with L-tyrosine had a significant difference of ($p < 0.0001$) compared to the control. L-serine also significantly increased bacterial inhibition but at a lower rate (Figure 1).

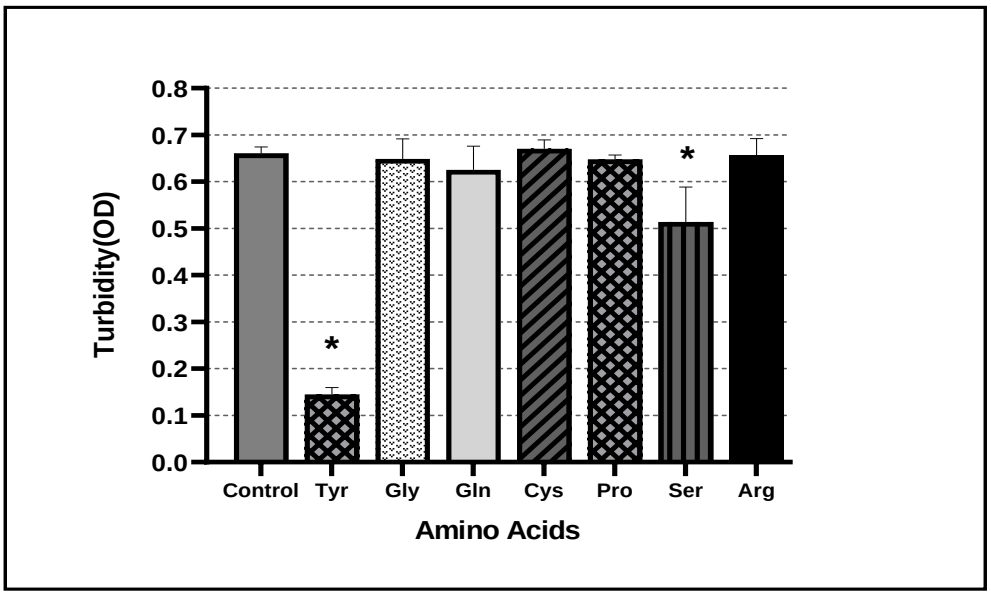

Figure 1: Effect of some amino acids on *P. mirabilis* growth. The turbidity of bacterial growth is represented on the y-axis (mean ± SD), and the challenge with amino acids is shown on the x-axis. *: Tyr and Ser had inhibitory effects with a significant difference $p< 0.05$.

Table 5 demonstrates a substantial decrease in the growth rate of the isolates treated with L-tyrosine which reached 0.1448 ± 0.01417 compared to the control where it was 0.6603 ± 0.01417. On the other hand, the inhibitory effect of L-serine on growth was considerably milder, showing a decrease of 0.5142 ± 0.07449 compared to the control. The reason for the inhibitory effect of L-tyrosine may arise from direct interactions with bacterial membranes, while the inhibitory effect of L-serine may be due to toxicity resulting from interference with the biosynthesis of threonine [20]. Further studies are needed to find out why these amino acids affect the growth of *Proteus mirabilis*. It is important to note that the specific inhibitory effects of amino acids on bacterial growth can vary depending on the bacterial species, strain, and environmental conditions. A study by Tawfeeq reported that L-tyrosine and L-serine do not have an inhibitory effect on the planktonic cells of *Staphylococcus aureus*, *Escherichia coli,* and *Klebsiella pneumonia* [21].

Table 5: The effect of amino acids on the growth of *P. mirabilis*

AAs	Turbidity (OD)		
	Mean	± SD	t-test**
Control	0.6603	0.01417	---
L-tyrosine	0.1448	0.01417	0.000*
Glycine	0.6485	0.0436	0.548^{ns}
L-glutamine	0.6255	0.05041	0.156^{ns}
L-cystine	0.6703	0.01899	0.326^{ns}
L-proline	0.6477	0.00969	0.101^{ns}
L-serine	0.5142	0.07449	0.004*
L-arginine	0.6555	0.03675	0.773^{ns}

**T-test: To compare every treatment with the control group. * ($p \leq 0.05$), ns: non-Significant.

Detection of *chABCI* Gene in *P. mirabilis*

Previous local studies did not detect the presence of the *chABCI* gene in *P. mirabilis*. However, it was identified in this species due to its more prevalence compared to *P. vulgaris* in Iraq. Based on the findings presented in Figure 2, it can be observed that the *chABCI* gene was present in five isolates of *P. mirabilis*, while the gene could not be detected in the *P. vulgaris* isolates. However, due to the limited number of common *P. vulgaris* isolated in this study, it was not possible to determine definitively whether the gene was present in *P. vulgaris* or not. All previous studies investigated its presence in *P. vulgaris* [16, 22]. However, there was no available local study about the detection of the *chABCI* gene in *P. mirabilis*.

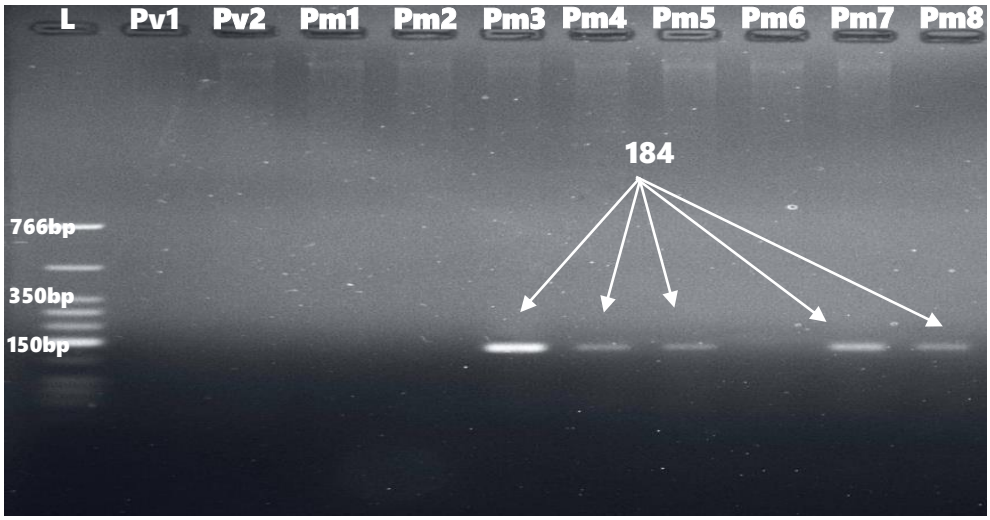

Figure 2: Agarose gel electrophoresis to detect the presence of the *chABCI* (Agarose: 2%, volt:80, for 80 min) Lane L: DNA ladder, other lanes represented the PCR product of *chABCI* gene with 184 base pairs of DNAs extracted from the isolates. Pm: *P. mirabilis*, Pv: *P. vulgaris*.

Alterations in Gene Expression Caused by Amino Acids

The findings illustrated in Figure 3 indicate that the gene expression of the target gene was significantly influenced ($p < 0.05$) by all the amino acids tested, as compared to the control. Each of these amino acids exhibited different degrees of increasing gene expression, except L-tyrosine which had the opposite effect. It showed an inhibitory effect which may be related to its effect on the growth of bacteria, as it was confirmed in this study that the growth of *P. mirabilis* was reduced. Other studies have proven an association with organism growth that can

be seen in the synthesis of enzymes [23]. Several studies suggested that gene expression changes frequently coincide with growth rate alterations [24, 25]. Consequently, it has been demonstrated that numerous amino acids manifest diverse growth-rate dependencies in terms of their expression.

The data showed that L-glutamine, glycine, and L-arginine had the highest upregulation effect on the expression of the *chABCI* gene, where the mean fold ranged from 19.7570 to 23.6653. It was followed by L-cystine and L-proline with folds of 10.3500 and 10.1902 respectively. L-serine also significantly increased the fold change but at a lower rate that did not exceed 4.1758, while L-tyrosine reduced (Downregulation) the gene expression of the *chABCI* to 0.2243.

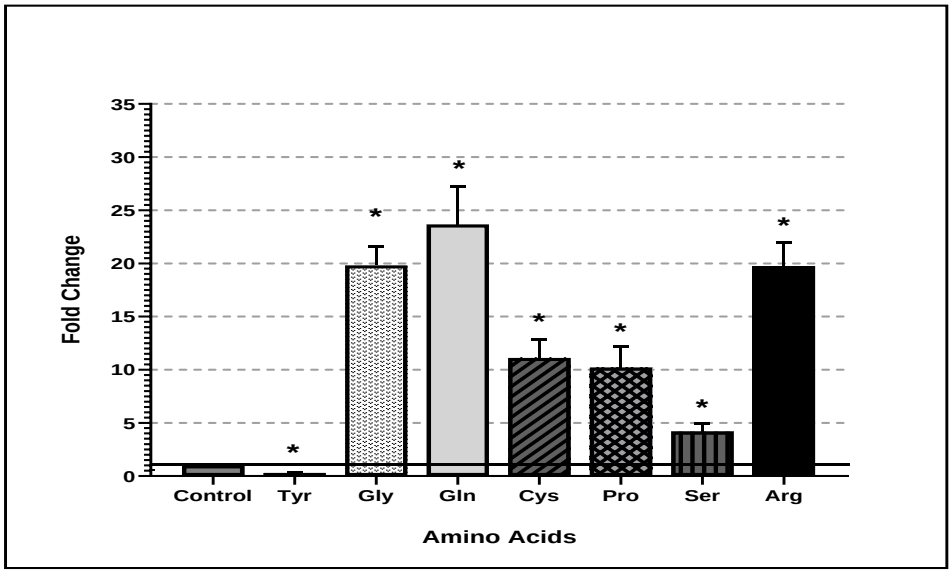

Figure 3: Effect of amino acids on the *chABCI* gene expression of *Proteus mirabilis*. * Indicates that the effect was significant.

Bacteria adjust their gene expression in response to environmental cues. Also, translational responses may result from modifications in nutrient concentrations within the cell [11]. There is still a limited understanding of how environmental trophic levels affect the genetic regulation of bacteria. Gene expression is mostly regulated at the transcription level which is regulated by RNA polymerase [26].

Upon take-up by bacteria, amino acids can be either straightforwardly consolidated into the bacterial cells as protein building blocks or catabolized. Amino acids are essential nutrients for the metabolism of carbon and nitrogen in bacteria. Changes in amino acid accessibility have a significant impact on a pathogen's proliferation and the expression of its virulence factor as the pathogen needs amino acids to support its physiological capabilities [23, 27]. Some amino acids have been used to find out their effect on the *chABCI* gene, whether they act as inducers or repressors.

There are no previous studies on the effect of amino acids on the *chABCI* gene but the findings by Penttinen *et al.,* who demonstrated that augmented nutrient concentration in the environment can increase (upregulation) the expression of chondroitinase (*cslA*) and collagenase in *Flavobacterium columnare* [28]. Other studies have proven that increased glutamine content intake considerably influences *Pasteurella multocida* frequency and gene expression, including *pm0442, pm0979, ompA, hasR*, and *plpE*. Similarly, glutamine controls

the expression of the virulence genes in *Listeria monocytogenes* by acting as an on/off switch. When glutamine levels in macrophages are below the threshold, *L. monocytogenes* does not transcribe any virulence genes. These findings show the significance of amino acids, particularly glutamine, in a pathogen's ability to proliferate and express virulence factors [27, 29]. However, it is unclear whether the virulence depends directly on L-glutamine or the availability of nitrogen in general.

Conclusion

L-tyrosine inhibited the growth of *P. mirabilis.* L-serine also showed inhibitory effects on growth but to a lesser extent than L-tyrosine. The gene expression of *chABCI* was upregulated significantly when treated with L-glutamine, glycine, L-arginine, L-proline, L-cysteine, and L-serine, while L-tyrosine had a down-regulation effect on the gene expression.

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Appendix

Figure for primer design, with the green annotation showing the primer binding site.