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IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF GENES INVOLVED IN PROLINE BIOSYNTHESIS IN EGYPTIAN SALT TOLERANT BACTERIAL ISOLATES

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ABSTRACT

In Egypt, the degradation of soil by salinity and alkalinity has been classified as a major agricultural problem especially in the northern part of the Nile Delta. This increase in the external salt concentration causes a serious problem to any living cells due to the loss of water. However, it leads to cell death if no countermeasures are taken. Some prokaryotes have developed different strategies to cope with increasing salinities by producing some amino acids such as proline, glycerol, glycine betaine, L-α- glutamate and ectoine. The present study was conducted on five isolates of salt tolerant bacteria that isolated from Egyptian solis. These isolates are producing proline to cope with the external salt stress. Three genes coding for: ornithine cyclodeaminase, proline iminopeptidase (pip) and pyrroline-5-carboxylate reductase (proC) were studied. The five isolates showed good growth in salt media up to (7.7 M NaCl). The microscopic examination in free media and T3 media showed thin and long vegetative cells, sporulated cells were thick and short and some crystal proteins began to appear. In salt media cells appeared with highly super-coiled shape. The existences of the three proline genes were 684 bp from pyrroline-5-carboxylate reductase gene, 757 bp from proline iminopeptidase gene, 674 bp from ornithine cyclodeaminase gene. Total protein pattern for all the isolates showed differences such as appearance of new bands between 72 kDa and 95 kDa in salt media. Proline was measured in all the isolates showing gradually increasing in proline concentration accompanied with regular increase in salinity treatment. The maximum proline concentrations was recorded at 1 M for isolate 2, 2.5 M for isolate 3, 2 M for isolate 4 and 5, and 1.5 M for isolate 6 of salt. The PCR products of the detected genes were sequenced and analyzed with NCBI BLAST program and they hit 19 of different *Bacillus thuringiensis*. One of them was *Bacillus thuringiensis* str. *Al Hakam* (CP000485.1). The sequences of isolate 2 produced significant alignments reached 99% for *pyrroline-5-carboxylate reductase* gene, 92% for proline iminopeptidase gene, and 91% for ornithine cyclodeaminase gene. Isolate 4 reached 99% for *pyrroline-5-carboxylate reductase* gene, 93 % for proline iminopeptidase gene, and 92% for ornithine cyclodeaminase gene. Isolate 5 reached 99 % for pyrroline-5-carboxylate reductase gene, 90% for proline iminopeptidase gene, and 91% for ornithine cyclodeaminase gene. Isolate 6 reached 94% pyrroline-5-carboxylate reductase gene, 94% for proline iminopeptidase gene, and 96% for ornithine cyclodeaminase gene.

Key words:*Bacillus thuringiensis*, salt stress, Ornithine cyclodeaminase, Proline iminopeptidase, Pyrroline-5-carboxylate reductase.

INTRODUCTION

Soil is a very complex system which includes a variety of microhabitats having different physicochemical gradients and microorganisms (Agnieszka *et al.*, 2013). Determining factors, like: temperature, pH, salinity, or geographic location indicated

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how simply microbes response to different environmental changes (Girvan *et al.*, 2003; Lozupone and Knight, 2007). Salinity is one of the major problems, particularly in arid and semi-arid regions, resulting in reducing crop production over the next 20 years (Nashwa *et al.*, 1991). Therefore; there is definitely need of upgrading drought and salinity tolerant crops (Mubshara *et al.*, 2012).

The bacteria exhibit in unusual extreme conditions such as salinity in fact may provide an important research tool for investigating the relationships and interactions between environmental factors and microorganism evolution at metabolic and gene levels (Darine *et al.*, 2009). There are many classes of halotolerant microbes: non-tolerant, those which tolerate only a low concentration of salt (about 1% w/v); slightly tolerant, tolerating up to 6-8%; moderately tolerant, up to 18-20%; and intensely tolerant, those microbes that grow over the total vary of salt concentrations from zero up to saturation (Sandhya and Harsha, 2012). It is well known that majority halophilic bacteria accumulate small organic compounds so-called compatible solutes, like glycine betaine, ectoine, proline, glutamate, and trehalose, to cope with external hyperosmotic conditions (Brice *et al.*, 2010).

Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and is crucial for primary metabolism that protects cells by stabilizing proteins and cellular membranes (Özge and Atak, 2012). Proline is one of the key osmolytes contributive toward osmotic adjustment (Soudry *et al.*, 2005 and Iqbal *et al.*, 2008) and its accumulation has been reported throughout conditions of drought and high salinity (Yoshiba *et al.*, 1995). A proline residue is found in all *Bacillus thuringiensis* (Bt) endotoxins, within the middle of an K-helix, and it`s supposed to be exist in more than 100 sequenced genes. This amino acid residue induces a broken-helix motif that confers a characteristic structure to domain 1 in these insecticidal toxins (Suzanne *et al.*, 2001).

The aim of this study was the identification and molecular characterization of proline genes in halophilic halotolerant Egyptian isolates of *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial Isolates

Five bacterial isolates of salt tolerant bacteria (isolates 2, 3, 4, 5 and 6) were obtained from Microbial Molecular Biology lab., AGERI (Agriculture Genetic Engineering Research Institute) that have been previously isolated from different soil locations in Egypt and identified as *Bacillus thuringiensis* (Nahed *et al.*, 2007).

Growth Conditions and Salt Treatments

The five isolates were grown in LB Broth (5 g yeast extract, 5 g sodium chloride and 10 g tryptone up to 1 litter), T3 medium (5 g Peptone, 1.5 g Yeast extract, 0.05 MnCl2 and 50 ml T3 buffer (0.5 m NaH_2Po_4 and 500 ml distilled H_2O, pH 6.8) up to 1 litter) and in salt media (LB media containing different concentrations of NaCl from 0 M to 7.7 M) to follow up its morphological appearance, crystal proteins and total cellular proteins differences in salt media by SDS-PAGE. When they were growing in LB media, they grew at 28°C, in T3 media at 37°C, and in salt media, at 37°C (Joanne and Ammons, 2005; Darine *et al.* 2009).

Protein Electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Li *et al.* (2002).

Proline Assay

Determination of proline was conducted according to Yoshiba *et al.* (1997).

DNA Extraction

DNA was extracted from the bacterial cells by boiling a single colony in 200 µl of distilled H_2O in a water bath for 10 min to lyses the cells followed with ice shock for 5 minutes, and then the tubes were centrifuged at 14.000 rpm to collect the condensate cells (Carozzi *et al*, 1991).

Specific PCR Reactions

Three pairs of specific primers that were used to amplify a 674 bp of ornithine cyclodeaminase gene (EC: 4.3.1.12), 757 bp of proline iminopeptidase gene (EC: 3.4.11.5), and

684 bp of pyrroline-5-carboxylate reductase gene (EC: 1.5.1.2) that are coding for proline amino acid synthesis were designed in MMB Lab. using NCBI PUBMED and synthesized by Lab Technology Company in Germany. The sequences of the three primers are illustrated bellow:

Pyrroline-5-carboxylate reductase (P5CR)

F 5′- TGGTGCTGGTCGTATGGCAGA -3′
R 5′-GCTGTGGAACCACCTGGCGT -3′

Proline iminopeptidase (pip)

F 5′-ACTTGCATGGTGGACCGGGAGA -3′
R5′TGAGGTGAATGACCGCTCTCTTCAAA-3′

Ornithine cyclodeaminase

F 5′-AACGGGAGAACCGCTCGCAC -3′
R 5′-CGCGCTCCACCGCTTTCTCA -3′

PCR reaction was performed in 25 µl total volume. Each PCR reaction contained 1X buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 2mM MgCl2, 0.01% (w/v) gelatin), 250µM each of dGTP, dATP, dCTP and dTTP, 2.5 units of Taq DNA polymerase, 100 pmol of each primer and the DNA template. Each PCR Eppendorf tubes contained the following:

Forward Primer	0.5µl
Reverse Primer	0.5µl
DNA Template	0.5µl
Taq polymerase (5u/µl) (containing MgCl2)	0.5µl
(10X)Taq polymerase Buffer	2.5µl
dNTPS (10 mM each)	0.5µl
d.dH2O	20µl

PCR Conditions

Amplification reactions were carried out using G-STORM DNA thermal cycler. The conditions were programmed as follows:

Heated Lid	110.0 °C	------
Hot start	94 °C	3 min
Start cycle	35 times	
Denaturation	94 °C	45 Sec
Annealing	62°C - 64.3°C - 65.1°C	1 min
Elongation	72°C	1 min
Elongation	72°C	7 min
Store	4°C	Infinite

10 µl from each PCR reaction was separated by agarose (1.2%) gel electrophoresis, stained with ethidium bromide (0.5µg/µl) and were run at 100 V in 1X TAE buffer and photographed on a UV transilluminator (BIO-RAD Gel Doc ™ XR+ with Image Lab Software) (Joanne and Ammons, 2005).

DNA Sequencing

PCR products were purified using QiaGen Qiaquick ® Kit, then sequenced by Lab Technology Company in Germany.

Sequence Analysis

Sequences results were alignment with data base ncbi nucleotide blast software (http:// blast. ncbi.nlm.nih.gov/Blast.cgi) (Chodak *et al.*, 2013).

RESULTS AND DISCUSSION

Colonial Morphology of Bacterial Isolates

The morphological characters of *B. thuringiensis* were detected and were constant as a white, round, flat colony, formed 'pan cake' like circular colonies with smooth or serrate margins with varying diameter as shown in Fig. 1 and were agreed with (Zakeel *et al.*, 2009; Thanalecthume *et al.*, 2011; Kannan *et al.*, 2012). Bt showed like a colonies with a round, white, with regular margins exhibiting identical shape and that was the same with the colonial morphology of the wild type *B. thuringiensis* strains but not as mentioned in Zakeel *et al.*, 2009 that appeared with as rhomboidal shape. Those characters are the same under salt stress and normal condition. However, there has been slower growth rate and slightly less for growth in normal circumstances, and that was observed in Fig. 1 B1, B2, B3 and B4.

Microscopic Examination of Bacterial Growth

During the microscopic examination, the bacterial candidates or vegetative cells are observed under the light microscope after gram staining as thin slender rods in short chains as illustrated in Fig. 2 A and B for isolates 4 and 5 for examples. In contrast, most cells in T3 medium showed the spores which appeared in green color and the toxin crystal proteins which were the most distinguishing characteristics of *Bt* in Fig. 2 C, D, E, F, G and H (marked by arrows) and that was agreed with (Kati *et al.*, 2007;

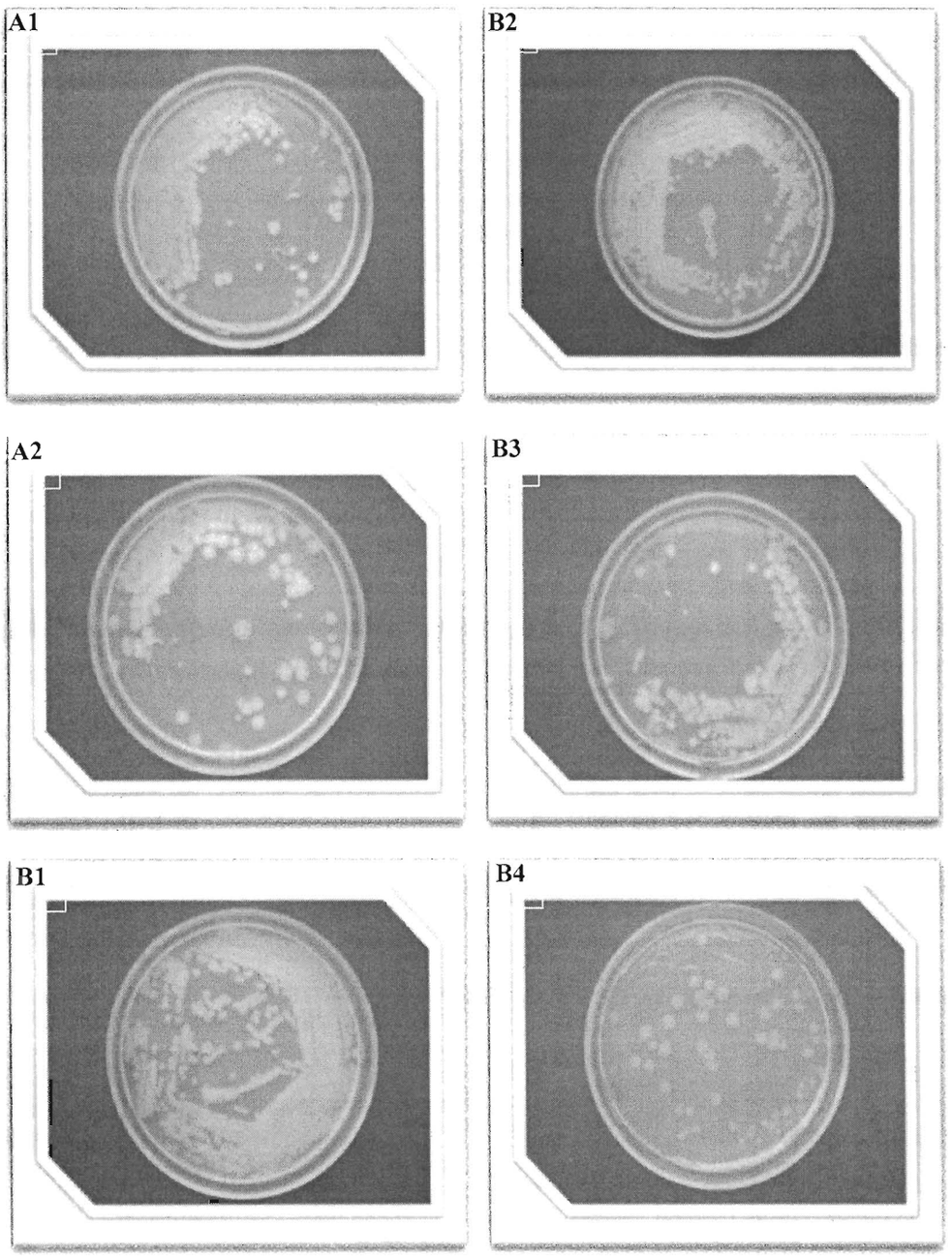


Fig.1. Photographs for the morphology of halotolerant isolates. A1: iso.4 in L.B agar free media; A2: iso.5 in L.B agar free media; B1: iso.4 in 1M of NaCl in L.B agar media; B2: iso.5 in 1M of NaCl in L.B agar media; B3: iso.4 in 1.5M of NaCl in L.B agar media; B4: iso.5 in 1.5M of NaCl in L.B agar media

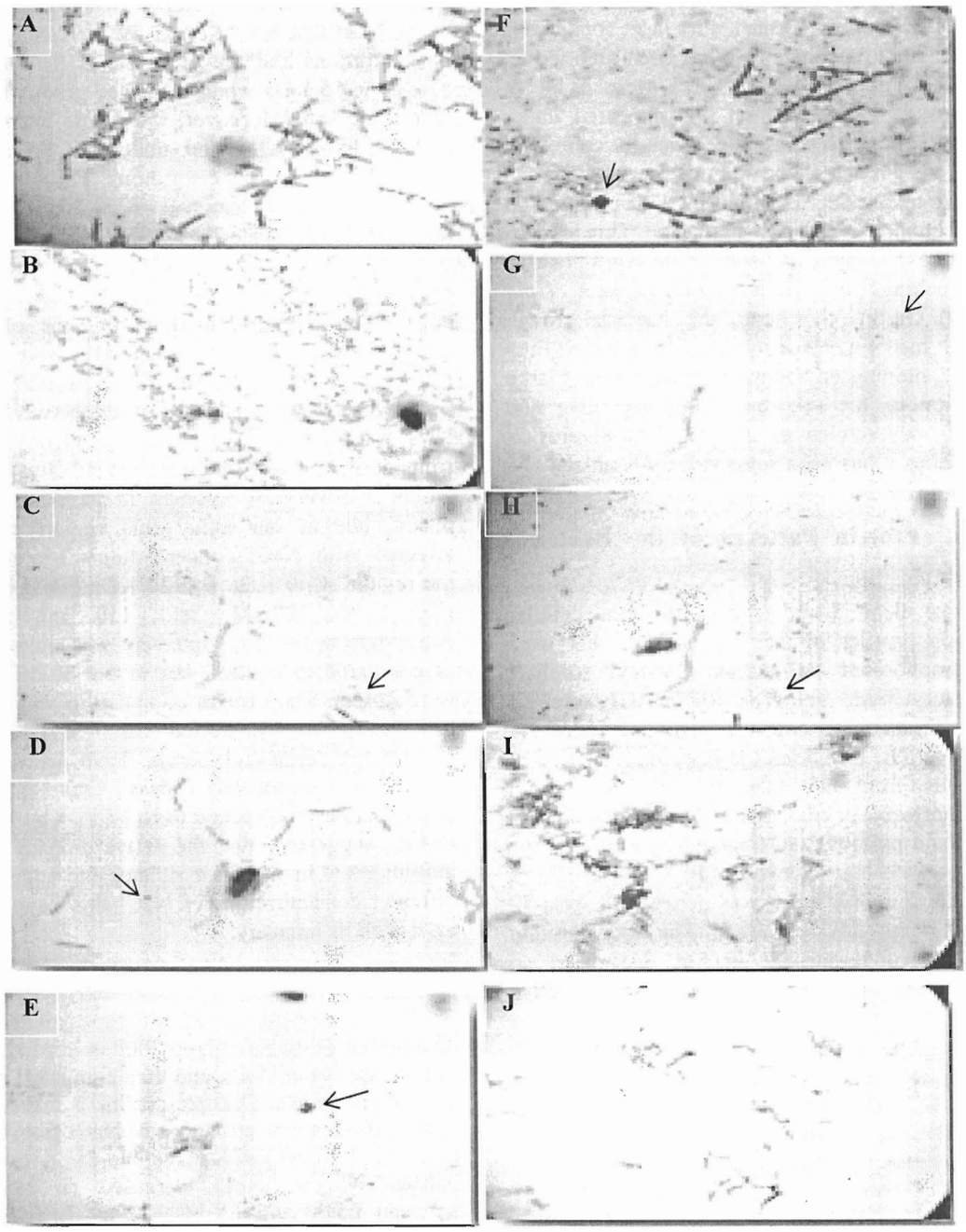


Fig. 2. The bacterial shape differed from normal case, in T3 medium, and under different concentration of NaCl. A and B show the fourth and the fifth isolates which were grown in L.B agar respectively, C, D and E was the fourth isolate that was grown in T3 medium for 3, 4 and 5 days and F, G, and H was the fifth isolate that was grown in T3 medium for 3, 4 and 5 days. I was the fourth isolate that was grown in medium contains 1M NaCl and J was the fifth isolate in the same medium

Thanalecthume *et al.*, 2011 and Kannan *et al.*, 2012). The spores formed were large round cells that failed to elongate as it has been reported by Malcolm *et al.* (2001). There were a lot of shapes of crystal protein that appeared under microscope such as square, triangle, cone, and even the circular shape and this is what has been agreed upon with Kati *et al.* (2007) as the crystal protein appeared with a flat shape. This assured that those isolates of bacteria are gram-positive and belongs to bacillus species (Zakeel *et al.*, 2009). On the other hand, the bacterial growth in salt media made distorted cells with the "live-dead" staining procedure and found that a large fraction of the deformed cells are alive and highly supercoiled as illustrated in Figures 2I and 2J as it has been suggested by Kathleen and Erhard (2012).

Total Protein Patterns of the Bacterial Isolates

Figs. 3, 4, 5 and 6 show the total cellular protein profiles of bacterial isolates that were electrophoresed on sodium dodecyl sulphate polyacrylamide gel. The SDS-PAGE analysis showed distinctly varying patterns. There are considerable differences in protein profiles of Bt isolates from 95 kDa to 28 kDa region (TusharKanti *et al.*, 2010). All isolates contain the main protein band (marked by arrow) at the top of each lane: between 130 kDa -95 kDa in isolates 2 and 3, but it was between 95 kDa-72 kDa in isolates 4 and 5. The total protein patterns of isolate 2 in Fig. 3 showed no significant differences between the protein patterns of the bacterial isolates in the different salt concentrations (from 1.7 M to 7.7 M of NaCl) in the region between 250 kDa and 28 KDa. On the other hand, there were significant faintness in the protein bands in the region between 28 KDa and 11 KDa, especially at high salt concentrations (5.1 M – 7.7 M). There were protein bands didn't change at all in salt concentrations as that found above the 55 KDa. The protein profile of isolate 3 in Fig. 4 showed main heavy band, between 130 and 95 KDa and was not affected by high concentrations of salt. The band at ~ 60 KDa is found at all salt concentrations in similarity to isolate 2. In Figs. 5 and 6 of isolates 4 and 5, the protein pattern of both of the them, showed high similarity in all concentrations of salt specifically the main band at 95 KDa that not affected by increasing salt concentrations and the other bands that found above the 55 KDa which vanished gradually by increasing salt. Moreover, the bands under 55 KDa ~ at 36 KDa and under 28 KDa not changed in all salt concentrations. These results are agreed with Muhammad *et al.*, 2013 results on plant leaves since they found that the total leaf extracted proteins content were gradually decreased with the increasing of NaCl concentrations in the medium in spite of the intensity of protein bands that came from total leaf extracted from free media. However, they found that the polypeptide bands between 40 KDa and 70 KDa was present in plant raised from smoke primed seeds but these bands were absent or of very low intensity in the salt treated plants, but in our study this region didn't affected with NaCl concentrations. Moreover, the region of 40 KDa and 20 KDa bands were present at 100mM, and 150 mM salt concentration but their intensity was quite low as compared to control, but in our study they were absent. The growth of isolate 6 was very low, so it was neglected from SDS-PAGE experiment. Jorge and Garcia, 1996 mentioned that the molecular mass of proline iminopeptidase was found to be 35 kDa by SDS-PAGE, suggesting that the active enzyme is a multimer and in all of our isolates and under the different concentrations of NaCl this band still exist with its intensity.

Determination of Proline

Proline content in all of the isolates is determined using NanoDrop 2000 at normal and salt-stressed conditions, and then compared with the control values. The free proline is measured under different concentrations of NaCl from 0 M to 5 M. Proline levels have increased in the isolates as the stress increased (it ranged between 1.5 M and 2.5 M) (Figs. 7, 8, 9, 10 and 11). This value increased in isolate 2 from 0.5 M to 1 M of NaCl to reach the maximum value of free proline in 1 M of NaCl. Isolate 3 was slightly different, free proline value increased from 0.5 M to 2.5 M of NaCl to reach the maximum value of free proline in 2.5 M of NaCl. Isolate 4 and 5 free proline values increased from 0.5 M to 2 M of NaCl to reach the maximum value of free proline in 2 M and in

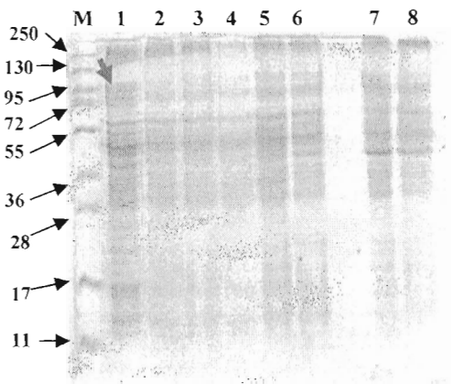


Fig. 3. SDS-PAGE of total bacterial protein for isolate 2. M: prestained protein marker, lane 1: bacterial protein of iso.2 in 1.7 M NaCl, lane 2: iso.2 in 2.6 M NaCl, lane 3: iso.2 in 3.4 M NaCl, lane 4: iso.2 in 4.3 M NaCl, lane 5: iso.2 in 5.1 M NaCl, lane 6: iso.2 in 6 M NaCl, lane 7: iso.2 in 6.8 M NaCl and lane 8: iso.2 in 7.7 M NaCl

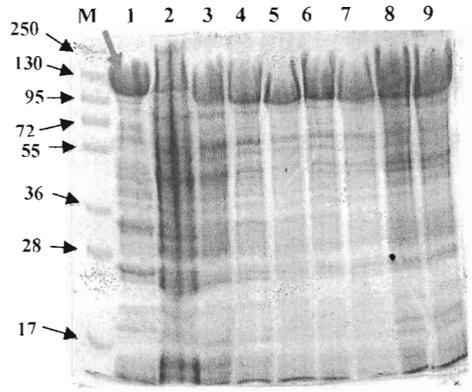


Fig. 4. SDS-PAGE of total bacterial protein for isolate 3. M: prestained protein marker, lane 1: bacterial protein of iso.3 in 0.8 M NaCl, lane 2: iso.3 in 1.7 M NaCl, lane 3: iso.3 in 2.6 M NaCl, lane 4: iso.3 in 3.4 M NaCl, lane 5: iso.3 in 4.3 M NaCl, lane 6: iso.3 in 5.1 M NaCl, lane 7: iso.3 in 6 M NaCl, lane 8: iso.3 in 6.8 M NaCl and lane 9: iso.3 in 7.7 M NaCl

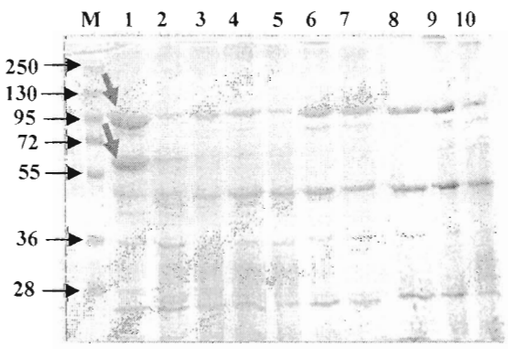


Fig. 5. SDS-PAGE of total bacterial protein for isolate 4. M: prestained protein marker, lane 1: bacterial protein of iso.4 in L.B normal media, lane 2: iso.4 in 0.85 M NaCl, lane 3: iso.4 in 1.7 M NaCl, lane 4: iso.4 in 2.6 M NaCl, lane 5: iso.4 in 3.4 M NaCl, lane 6: iso.4 in 4.3 M NaCl, lane 7: iso.4 in 5.1 M NaCl lane 8: iso.4 in 6 M NaCl, lane 9: iso.4 in 6.8 M NaCl and lane 10: iso.4 in 7.7 M NaCl

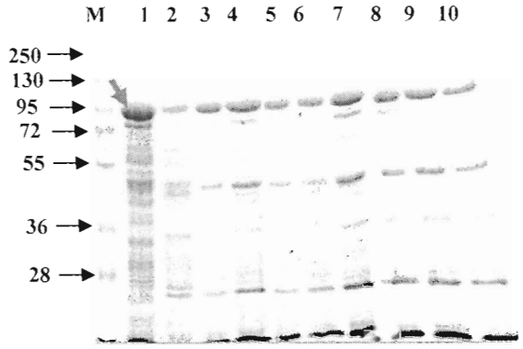


Fig. 6. SDS-PAGE of total bacterial protein for isolate 5. M: prestained protein marker, lane 1: bacterial protein of iso.5 in L.B normal media, lane 2: iso.5 in 0.85 M NaCl, lane 3: iso.5 in 1.7 M NaCl, lane 4: iso.5 in 2.6 M NaCl, lane 5: iso.5 in 3.4 M NaCl, lane 6: iso.5 in 4.3 M NaCl, lane 7: iso.5 in 5.1 M NaCl lane 8: iso.5 in 6 M NaCl, lane 9: iso.5 in 6.8 M NaCl and lane 10: iso.5 in 7.7. M NaCl

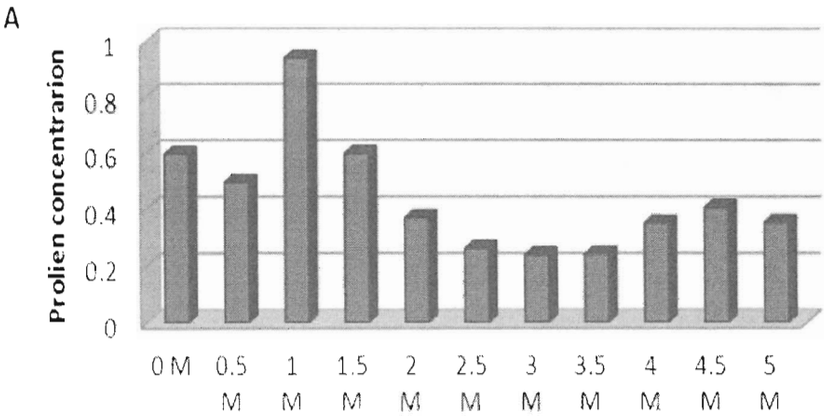


Fig.7. Proline curve with isolate 2 under 0 M, 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M, 3.5 M, 4 M, 4.5 M and 5 M of NaCl compared with the control of L-proline

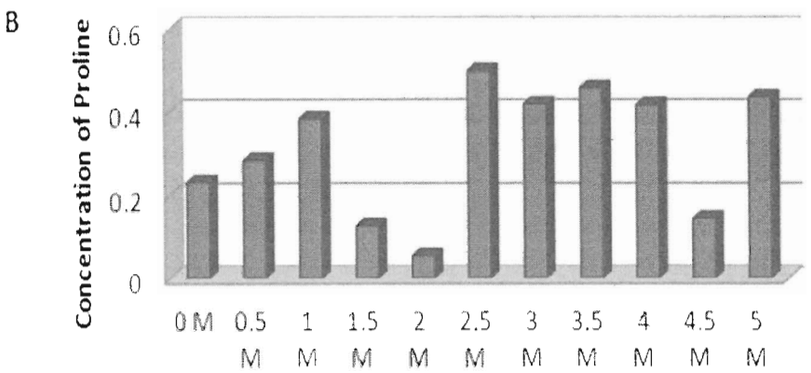


Fig. 8. Proline curve with isolate 3 under 0 M, 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M, 3.5 M, 4 M, 4.5 M and 5 M of NaCl compared with the control of L-proline

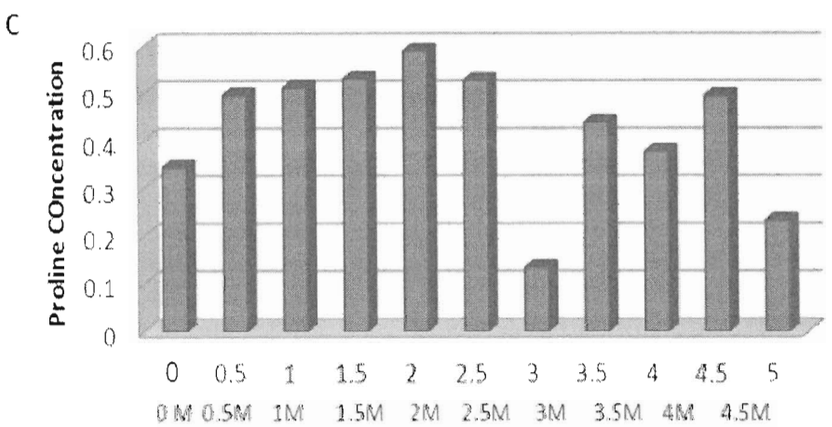


Fig. 9. Proline curve with isolate 4 under 0 M, 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M, 3.5 M, 4 M, 4.5 M and 5 M of NaCl compared with the control of L-proline

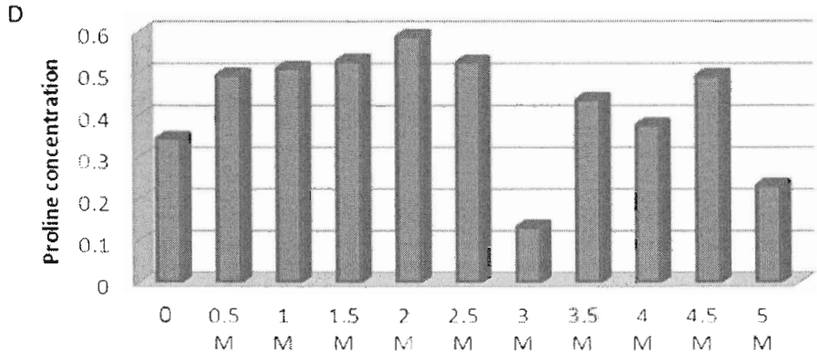


Fig. 10. Proline curve with isolate 5 under 0 M, 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M, 3.5 M, 4 M, 4.5 M and 5 M of NaCl compared with the control of L-proline

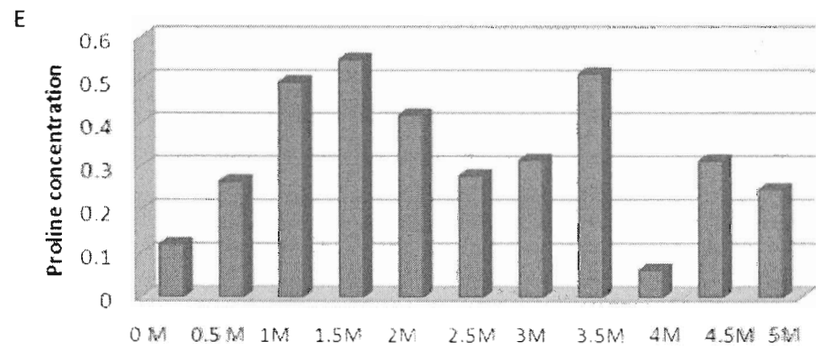


Fig.11. Proline curve with isolate 6 under 0 M, 0.5 M, 1 M, 1.5 M, 2 M, 2.5 M, 3 M, 3.5 M, 4 M, 4.5 M and 5 M of NaCl compared with the control of L-proline

isolate 6 from 0.5 M to 1.5 M of NaCl to reach the maximum value of free proline in 1.5 M and that was compatible with Marin *et al.* (2010) and Heshmat *et al.* (2012) who reported that proline has been increased with the stress, and was very high at 180 mM NaCl. That was somewhat different with (Killhamt and Firestone, 1984) who reported that proline increased from less than 6% of the free amino acid pool in cells grown in basal medium to about 50% of the pool in cells challenged with 1 M salt. Also there are some differences in proline amino acid that have been isolated from plant. Proline began to be produced in leaves from 50 to 100 mM NaCl showed statistically significant differences in the in vitro salinity experiment compared with leaves from controls as it have been observed by (Mahamadou *et al.*, 2013).

PCR Detection of Proline Genes

To detect proline genes, primers were synthesized at MMB lab on more than one step. The first step was to find the accession number of each gene, and we found them through our survey on the arginine and proline metabolism of *Bacillus thuringiensis* Al Hakam that showed in Fig. 12. There were three genes involving in production of proline amino acid: Pyrroline-5-carboxylate reductase (EC: 1.5.1.2), proline iminopeptidase (EC: 3.4.11.5) and ornithine cyclodeaminase (EC: 4.3.1.12). The molecular weights of the three genes were: 804 bp, 813 bp and 978 bp respectively. Then, NCBI (Primer-BLAST) tools were used for designing three pairs of specific primers, pair for each gene. Each pair was chosen carefully according to the ideal nucleotides length for primers (about 20 nucleotides), the GC ratio (about 50%), the differences between the Tm's of the forward and reverse (must be similar or at most 2°C differences) and the most important parameters are the primer-dimers and the product length as they were chosen with no primer-dimers and for

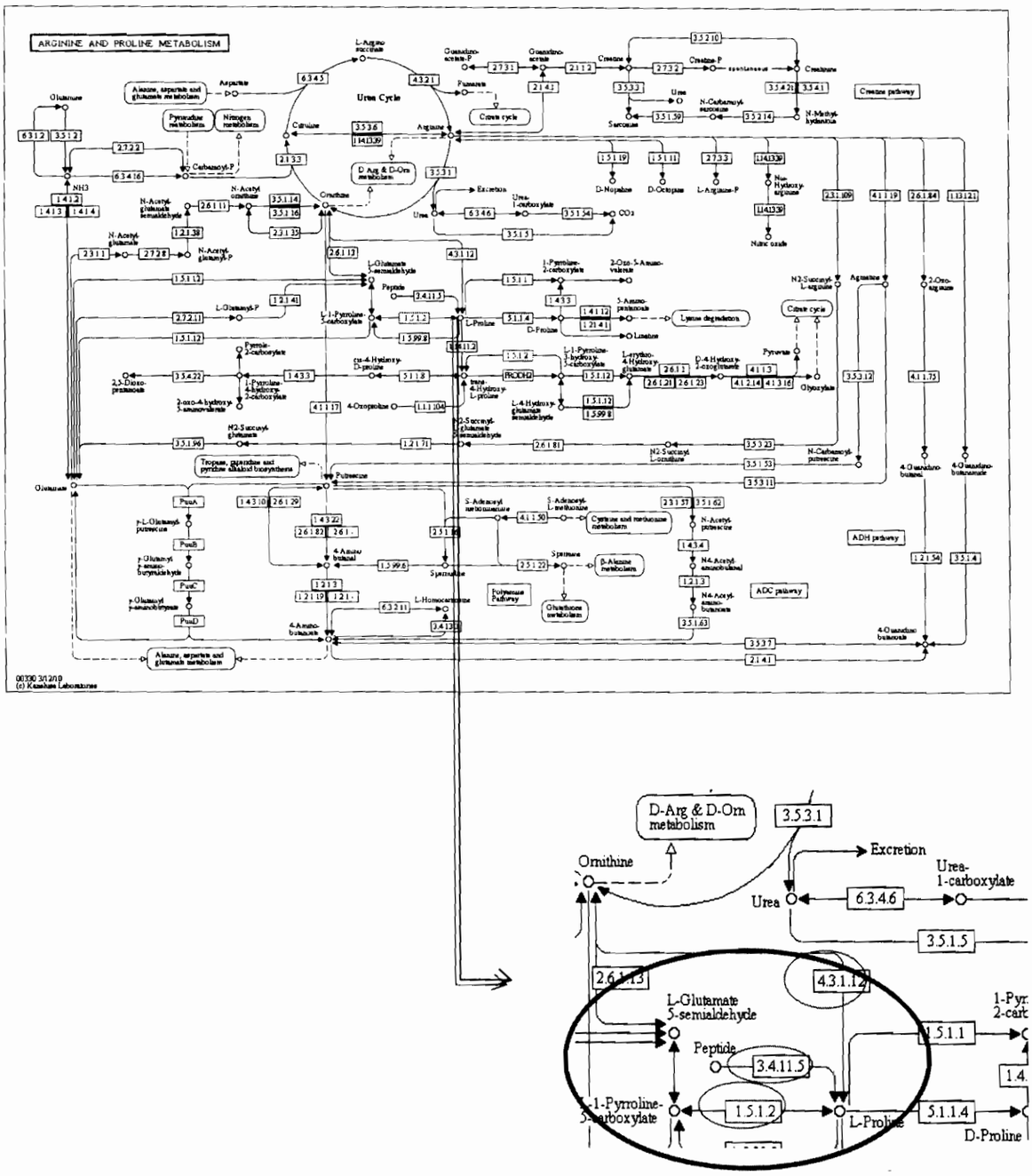


Fig. 12. Arginine and proline metabolism in *Bacillus thuringiensis* Al Hakam. The three genes; pyrroline-5-carboxylate reductase, proline iminopeptidase and ornithine cyclodeaminase that involved in the proline production in *Bacillus thuringiensis* Al Hakam are showed between the red circle tacking the EC no.(s), 1.5.1.2, 3.4.11.5 and 4.3.1.12 respectively

(http://www.genome.jp/kegg-bin/show_pathway?map00330)

the longest product length for each gene. The first pair of primer was designed for Pyrroline-5-carboxylate reductase gene to give 684 bp product length, the second was for proline iminopeptidase gene to give 757 bp and the third was for ornithine cyclodeaminase gene to give 674 bp. PCR was conducted for each gene with all the isolates and the three genes were identified in all the isolates except isolate 3, which gave a different product length with the three primers. However, it showed tolerance to salt stress and proline production as illustrated in Figs. 13, 14 and 15. Finally, the obtained PCR products were confirmed from the sequencing and the alignment of each DNA product.

DNA Sequencing and Alignment

PCR products were sequenced in LAB TECHNOLOGY Company in Germany. Then they were searched against the database (www.ncbi.nlm.com) using BLAST tool and they gave revealed homologs with Bt and the same three genes with results ranged between 81% to 97%. BLAST analysis confident our results. All belonged to *B.thuringiensis* and the same three genes. For example, (16 and 17) the alignment result of isolate 6 forward and reverse with primer 2 gave results cut off of 97% similarity and nocked 27 hits blots with *B. thuringiensis* such as: *Bacillus thuringiensis* str. Al Hakam, *Bacillus thuringiensis serovar thuringiensis str. IS5056*, *Bacillus thuringiensis BMB17* and *Bacillus thuringiensis Bt407*, as it has been indicated by Talat *et al.* (2012). Furthermore, multiple sequence alignment was carried out for all sequences results from each gene to discriminate and compare those sequences among each other. As illustrated in Figure 18, the multiple sequence alignment graph that generated from the fragments hat came from isolates 2, 4, 5 and 6 with primer 2 and proline iminopeptidase gene reflected a high degree of DNA relatedness among them (Talat *et al.*, 2012).

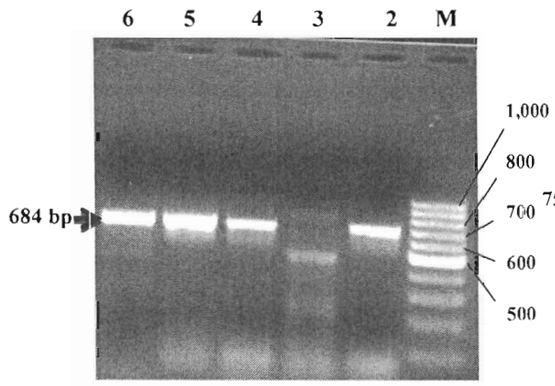


Fig. 13. PCR amplified product of x gene with primer 1. Lane M: DNA molecular marker 100 bp ladder. Lane 2: PCR product from detected gene in isol.2, lane 3: PCR product from detected gene in iso.3, lane 4: PCR product from detected gene in isol.4, lane 5: PCR product from detected gene in isol.5, and lane 6: PCR product from detected gene in isol.6. The four isolates 2, 4, 5 and 6 are showing the same band of 684 bp

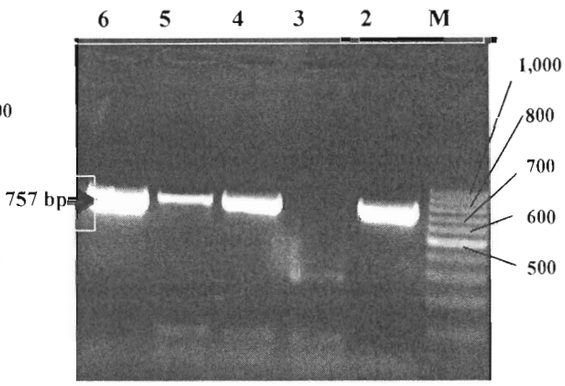


Fig. 14. PCR amplified product of proline iminopeptidase gene with primer 2. Lane M: DNA molecular marker 100 bp ladder. Lane 2: PCR product from detected gene in isol.2, lane 3: PCR product from detected gene in iso.3, lane 4: PCR product from detected gene in isol.4, lane 5: PCR product from detected gene in isol.5, and lane 6: PCR product from detected gene in isol.6. The four isolates 2, 4, 5 and 6 are showing the same band of 757 bp

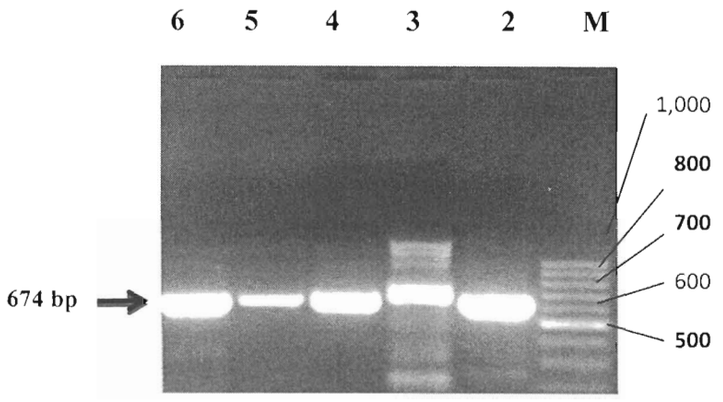


Fig.15.PCR amplified product of ornithine cyclodeaminase gene with primer 3. Lane M: Fermentas ladder of 100 bp was used as a standard size maker. Lane 2: PCR product from detected gene in isol.2, lane 3: PCR product from detected gene in iso.3, lane 4: PCR product from detected gene in isol.4, lane 5: PCR product from detected gene in isol.5, and lane 6: PCR product from detected gene in isol.6. The four isolates 2, 4, 5 and 6 are showing the same band of 674 bp

Sequences producing significant alignments:

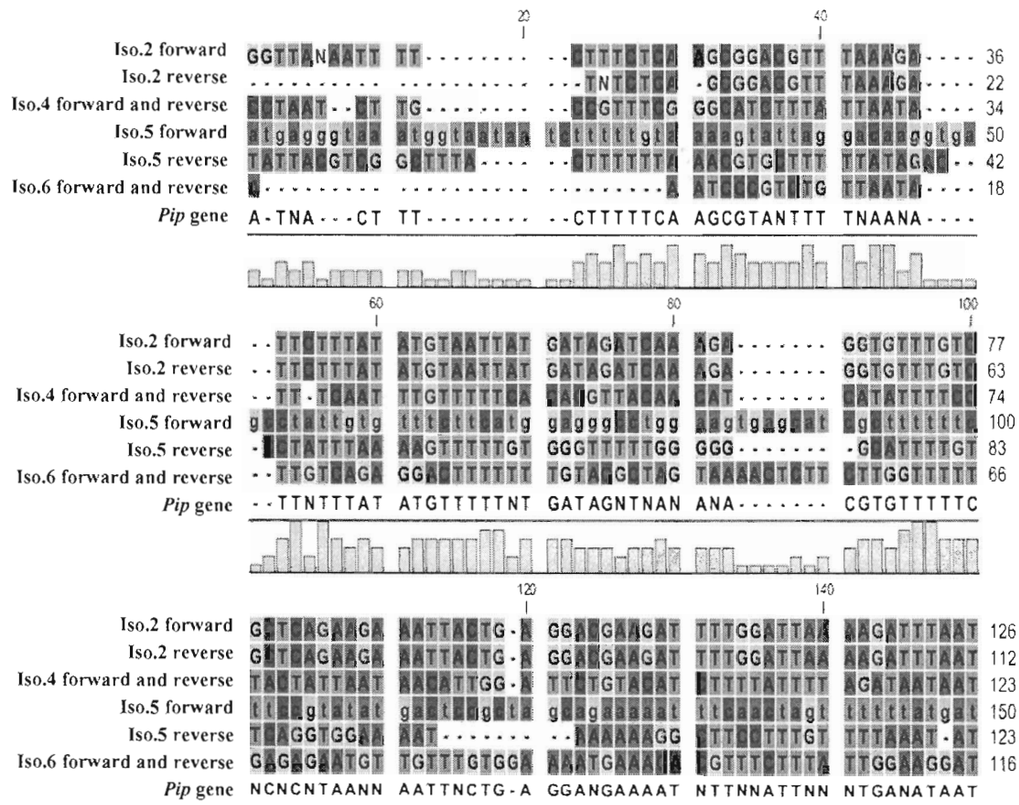
Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
YP_003664485.1	proline iminopeptidase [Bacillus thuringiensis BMB171] >gb|ADH067	348	348	97%	3e-96	85%	G
ZP_04114633.1	hypothetical protein bthur0006_19550 [Bacillus thuringiensis serovar	347	347	97%	4e-96	85%	
ZP_04120208.1	hypothetical protein bthur0005_19940 [Bacillus thuringiensis serovar	346	346	97%	1e-95	85%	
ZP_00742852.1	Proline iminopeptidase [Bacillus thuringiensis serovar israelensis ATC	343	343	97%	8e-95	84%	
ZP_04065023.1	hypothetical protein bthur0014_20100 [Bacillus thuringiensis IBL 422	341	341	97%	3e-94	84%	
ZP_04084268.1	hypothetical protein bthur0011_19410 [Bacillus thuringiensis serovar	340	340	97%	7e-94	83%	
ZP_04071799.1	hypothetical protein bthur0013_21120 [Bacillus thuringiensis IBL 200	335	335	97%	2e-92	82%	
YP_894799.1	proline iminopeptidase [Bacillus thuringiensis str. Al Hakam] >gb|AB	325	325	97%	2e-89	79%	G
YP_036330.1	proline iminopeptidase [Bacillus thuringiensis serovar konkukian str.	325	325	97%	2e-89	79%	G
ZP_04078429.1	hypothetical protein bthur0012_20501 [Bacillus thuringiensis serovar	324	324	97%	4e-89	79%	
ZP_04096274.1	hypothetical protein bthur0009_19880 [Bacillus thuringiensis serovar	320	320	97%	6e-88	78%	
ZP_04145469.1	hypothetical protein bthur0001_20060 [Bacillus thuringiensis serovar	317	317	97%	5e-87	78%	
ZP_04101941.1	hypothetical protein bthur0008_20100 [Bacillus thuringiensis serovar	314	314	97%	4e-86	76%	
ZP_04126278.1	hypothetical protein bthur0004_20210 [Bacillus thuringiensis serovar	197	197	45%	6e-51	91%	
ZP_04126279.1	hypothetical protein bthur0004_20220 [Bacillus thuringiensis serovar	139	139	42%	3e-33	73%	
ZP_04065205.1	Proline iminopeptidase [Bacillus thuringiensis IBL 4222] >gb|EEN031	73.9	73.9	33%	1e-13	38%	
YP_899828.1	proline iminopeptidase [Bacillus thuringiensis str. Al Hakam] >gb|AB	54.7	54.7	42%	9e-08	31%	G
ZP_04095352.1	Proline iminopeptidase [Bacillus thuringiensis serovar andalousiensis	52.0	52.0	30%	5e-07	32%	
ZP_04078893.1	hypothetical protein bthur0012_25200 [Bacillus thuringiensis serovar	50.1	50.1	30%	2e-06	32%	
ZP_04090791.1	hypothetical protein bthur0010_24480 [Bacillus thuringiensis serovar	50.1	50.1	30%	2e-06	32%	
ZP_04108632.1	hypothetical protein bthur0007_24520 [Bacillus thuringiensis serovar	50.1	50.1	30%	2e-06	32%	
YP_895215.1	alpha/beta fold family hydrolase [Bacillus thuringiensis str. Al Hakam	50.1	50.1	30%	2e-06	32%	G
ZP_04101622.1	Prolyl aminopeptidase (Proline iminopeptidase) [Bacillus thuringiensi	49.3	49.3	29%	3e-06	38%	
YP_896960.1	prolyl aminopeptidase [Bacillus thuringiensis str. Al Hakam] >gb|ABI	48.5	48.5	29%	6e-06	31%	G
ZP_04096816.1	hypothetical protein bthur0009_24330 [Bacillus thuringiensis serovar	48.1	48.1	30%	7e-06	32%	
ZP_04086710.1	hypothetical protein bthur0011_44010 [Bacillus thuringiensis serovar	47.8	47.8	29%	1e-05	31%	
ZP_04116958.1	hypothetical protein bthur0006_43060 [Bacillus thuringiensis serovar	47.8	47.8	29%	1e-05	31%	

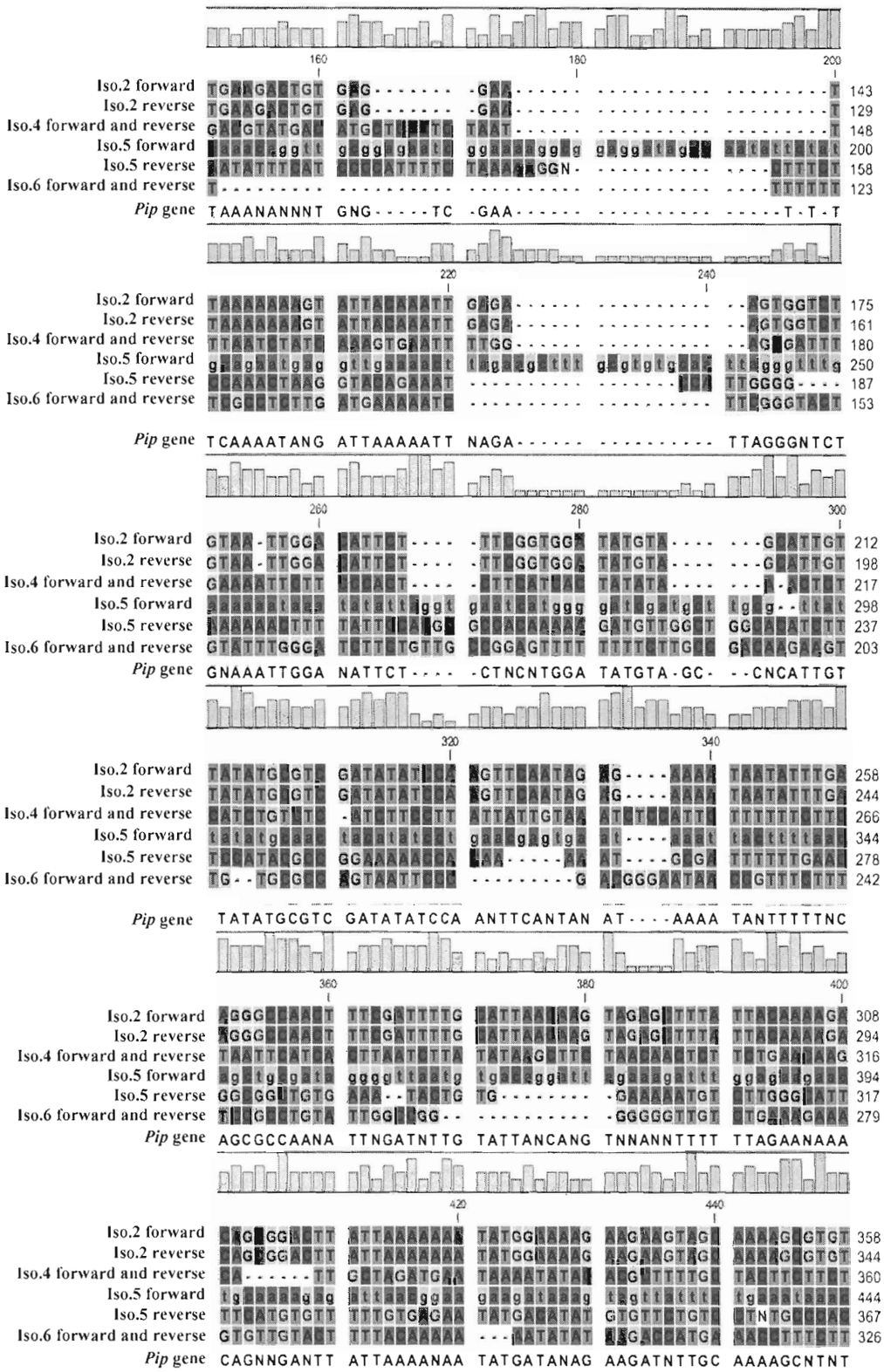
Fig. 16. Alignment using NCBI BLAST tools for sequenced PCR product with F primer 2 that came from isolate 6 and proline iminopeptidase gene in data base

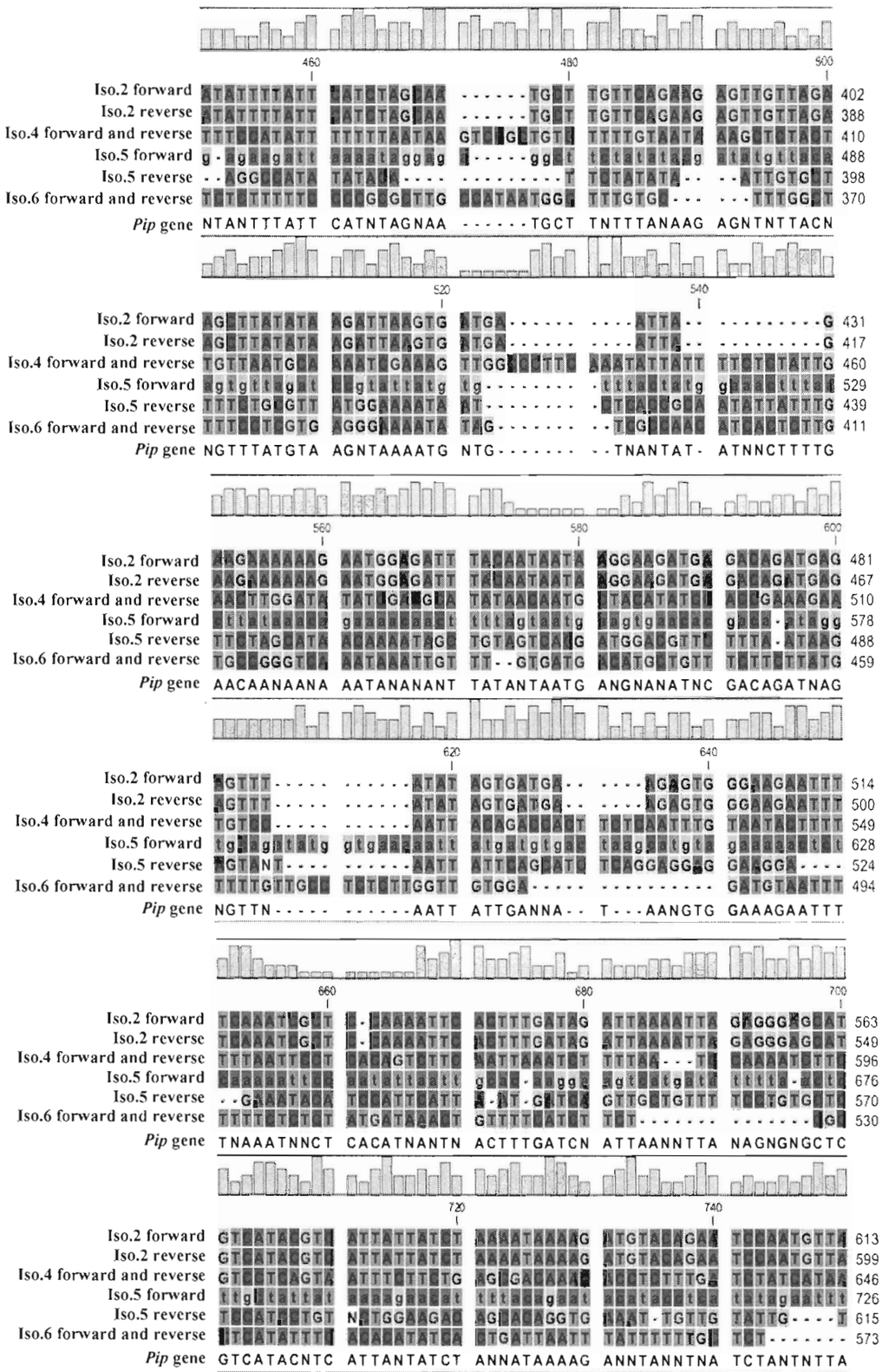
Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
YP_003664485.1	proline iminopeptidase [Bacillus thuringiensis BMB171] >gb|ADH067(	426	426	97%	1e-119	97%	G
ZP_04114633.1	hypothetical protein bthur0006_19550 [Bacillus thuringiensis serovar	426	426	97%	1e-119	97%	
ZP_04120208.1	hypothetical protein bthur0005_19940 [Bacillus thuringiensis serovar	424	424	97%	4e-119	96%	
ZP_04065023.1	hypothetical protein bthur0014_20100 [Bacillus thuringiensis IBL 422	419	419	97%	9e-118	95%	
ZP_04084268.1	hypothetical protein bthur0011_19410 [Bacillus thuringiensis serovar	416	416	97%	1e-116	95%	
ZP_04071799.1	hypothetical protein bthur0013_21120 [Bacillus thuringiensis IBL 200	414	414	97%	5e-116	94%	
ZP_00742352.1	Proline iminopeptidase [Bacillus thuringiensis serovar israelensis ATC	412	412	97%	2e-115	94%	
YP_894799.1	proline iminopeptidase [Bacillus thuringiensis str. Al Hakam] >gb|AB	397	397	97%	6e-111	90%	G
ZP_04078429.1	hypothetical protein bthur0012_20500 [Bacillus thuringiensis serovar	395	395	97%	1e-110	89%	
YP_036330.1	proline iminopeptidase [Bacillus thuringiensis serovar konkukian str.	394	394	97%	3e-110	89%	G
ZP_04096374.1	hypothetical protein bthur0009_19880 [Bacillus thuringiensis serovar	392	392	97%	2e-109	88%	
ZP_04145469.1	hypothetical protein bthur0001_20060 [Bacillus thuringiensis serovar	391	391	97%	3e-109	89%	
ZP_04101941.1	hypothetical protein bthur0008_20100 [Bacillus thuringiensis serovar	389	389	97%	1e-108	87%	
ZP_04126278.1	hypothetical protein bthur0004_20210 [Bacillus thuringiensis serovar	228	228	49%	4e-60	95%	
ZP_04126279.1	hypothetical protein bthur0004_20220 [Bacillus thuringiensis serovar	186	186	39%	2e-47	95%	
ZP_04065205.1	Proline iminopeptidase [Bacillus thuringiensis IBL 4222] >gb|EEN031	77.4	77.4	39%	1e-14	37%	
YP_893828.1	proline iminopeptidase [Bacillus thuringiensis str. Al Hakam] >gb|AB	54.7	54.7	42%	8e-08	31%	G
ZP_04095352.1	Proline iminopeptidase [Bacillus thuringiensis serovar andalousiensis	52.0	52.0	30%	5e-07	32%	
YP_896960.1	prolyl aminopeptidase [Bacillus thuringiensis str. Al Hakam] >gb|ABI	51.6	51.6	88%	7e-07	22%	G
ZP_04078893.1	hypothetical protein bthur0012_25200 [Bacillus thuringiensis serovar	50.4	50.4	33%	1e-06	32%	
ZP_04090791.1	hypothetical protein bthur0010_24480 [Bacillus thuringiensis serovar	50.4	50.4	33%	1e-06	32%	
ZP_04108632.1	hypothetical protein bthur0007_24520 [Bacillus thuringiensis serovar	50.4	50.4	33%	1e-06	32%	
YP_895215.1	alpha/beta fold family hydrolase [Bacillus thuringiensis str. Al Hakam	50.4	50.4	33%	1e-06	32%	G
ZP_04101622.1	Prolyl aminopeptidase (Proline iminopeptidase) [Bacillus thuringiensi	49.3	49.3	29%	3e-06	38%	
ZP_04096816.1	hypothetical protein bthur0009_24330 [Bacillus thuringiensis serovar	48.5	48.5	33%	6e-06	32%	
ZP_04086710.1	hypothetical protein bthur0011_44010 [Bacillus thuringiensis serovar	48.1	48.1	88%	7e-06	21%	
ZP_04116958.1	hypothetical protein bthur0006_43060 [Bacillus thuringiensis serovar	47.8	47.8	29%	9e-06	31%	

Fig. 17. Alignment using NCBI BLAST tools for sequenced PCR product with R primer 2 that came from isolate 6 and proline iminopeptidase gene in data base




160
180
200
Iso.2 forward
Iso.2 reverse
Iso.4 forward and reverse
Iso.5 forward
Iso.5 reverse
Iso.6 forward and reverse
Pip gene
TAAANANNNT GNG-----TC -GAA---------- ----------T-T-T
220
240
260
280
300
TCAAAATANG ATTAAAAATT NAGA---------- TTAGGGNTCT
320
340
GNAAATTGGA NATTCT---- -CTNCNTGGA TATGTA-GC- --CNCATTGT
360
380
400
TATATGCGTC GATATATCCA ANTTCANTAN AT----AAAA TANTTTTTNC
420
440
AGCGCCAANA TTNGATNTTG TATTANCANG TNNANNTTTT TTAGAANAAA
CAGNNGANTT ATTAAAANAA TATGATANAG AAGATNTTGC AAAAGCNTNT


460
480
500
Iso.2 forward
Iso.2 reverse
Iso.4 forward and reverse
Iso.5 forward
Iso.5 reverse
Iso.6 forward and reverse
Pip gene
NTANTTTATT CATNTAGNAA ------TGCT TNTTTANAAG AGNTNTTACN
520
540
NGTTTATGTA AGNTAAAATG NTG------ --TNANTAT- ATNNCTTTTG
560
580
600
AACAANAANA AATANANANT TATANTAATG ANGNANATNC GACAGATNAG
620
640
NGTTN----- ------AATT ATTGANNA-- T---AANGTG GAAAGAATTT
660
680
700
TNAAATNNCT CACATNANTN ACTTTGATCN ATTAANNTTA NAGNGNGCTC
720
740
GTCATACNTC ATTANTATCT ANNATAAAAG ANNTANNTNA TCTANTNTTA

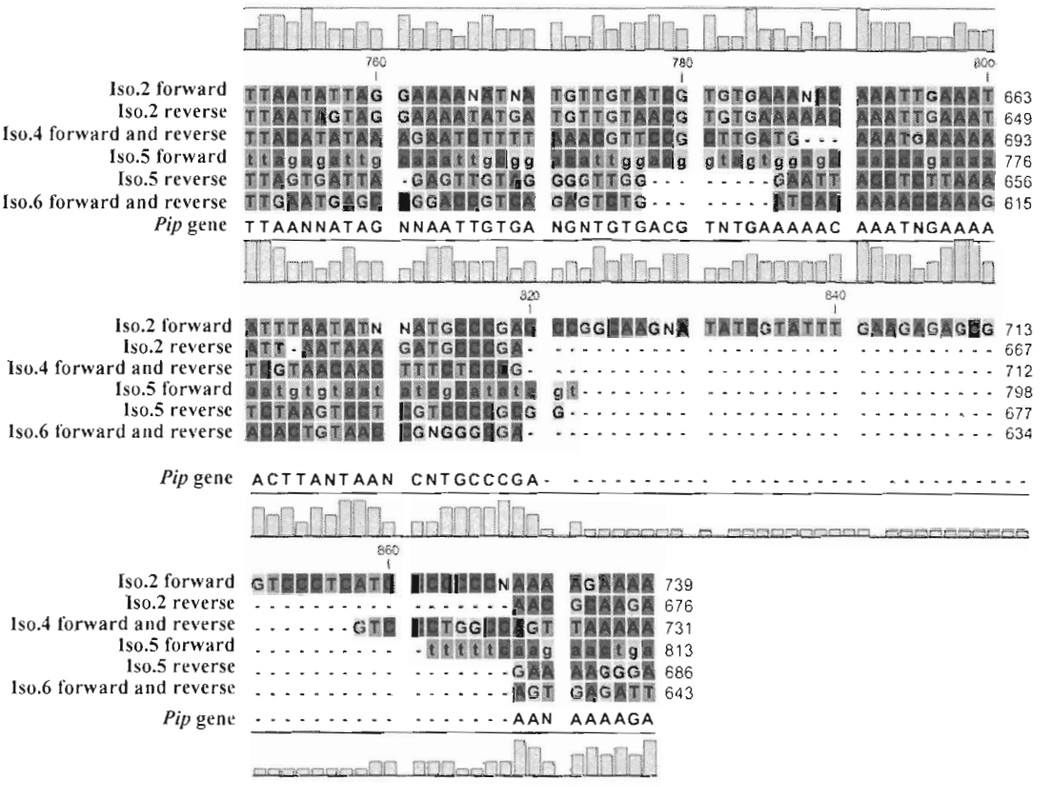


Fig. 18. Multiple sequence alignment for proline iminopeptidase gene sequence and sequenced PCR fragments that came from the four isolates: isolate 2 (F and R with primer 2 results), isolate 4 (the conting sequence of the F and R with primer 2 results), isolate 5 (F and R with primer 2 results) and isolate 6 (the conting sequence of the F and R with primer 2 results)

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التحديد والتوصيف الجزيئى للجينات المشاركة فى إنتاج البرولين فى البكتريا المتحملة للملوحة المعزولة من الأراضى المصرية

مى محمد لبيب [١] – أحمد شوقى حسن [٢] - ممدوح كامل أمين [٢] – ناهد عبد الغفار إبراهيم [١]

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الأراضى الملحية فى مصر تعتبر من أهم المشكلات الزراعية الرئيسية وخاصة في الجزء الشمالي من دلتا النيل، فالزيادة في تركيز الأملاح الخارجية تسبب مشاكل خطيرة للتربه و لأي كائن حى ويرجع ذلك إلى فقدان الماء مما يؤدى فى النهايه الى موت الخلايا و تلف التربه إذا لم يتم اتخاذ التدابير المضادة، وقد وضعت بعض بدائيات النوى استراتيجيات مختلفة للتعامل مع زيادة الملوحة من خلال إنتاج بعض الأحماض الأمينية مثل ال glycerol, glycine betaine, proline, ectoine , L-α- glutamate. لقد أجريت هذه الدراسة على خمسة عزلات من البكتيريا المعزولة من الأراضى الملحية المصرية، هذه العزلات لها القدرة على إنتاج البرولين كحمض امينى للتعامل مع زيادة نسبة الأملاح الخارجية. هناك ثلاث جينات مسئولة عن إنتاج البرولين فى بكتريا ال *Bacillus thuringiensis* وهم: ornithine cyclodeaminase وproline iminopeptidase وpyrroline-5-carboxylate reductase، بداية لقد تم تنمية هذه العزلات فى بيئات ملحيه بتركيزات مختلفة حتى وصلت إلى ٧,٧ مولر من ال NaCl وأظهرت العزلات نمو جيد فيها، أظهر الفحص المجهري تحت الميكروسكوب الخلايا البكتيرية فى بيئة ال L.B و ال T3 كخلايا رفيعة وطويلة كما ظهرت ال crystal proteins فى بيئة ال T3، و لكن فى البيئات الملحية ظهرت الخلايا سميكة وقصيرة. لقد تم عمل Protein gel electrophoresis للعزلات وأوضحت وجود اختلافات بين العزلات وبعضها وأيضاً داخل كل عزله تحت تأثير التركيزات المختلفة من الأملاح، أيضا تم قياس تركيز البرولين كحامض أمينى لكل العزلات وتحت التركيزات المختلفة من الأملاح وتم تسجيل أعلى تركيز من الأملاح الذى عنده تم إنتاج أعلى نسبه من البرولين لكل عزله، كذلك تم عمل Specific PCR لكل العزلات للثلاث جينات و لقد حصلنا على ال specific band لكل العزلات مع الثلاث جينات فيما عدا العزلة رقم ٣ حيث أنها أعطت band مختلفة، و فى النهاية تم عمل Sequencing ثم Alignment باستخدام ال NCBI Blast tools حيث أعطوا نسبه تشابه مع نفس الجينات و مع نفس البكتريا بنسبه تراوحت من ٩١% إلى ٩٩%.

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