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RESEARCH ARTICLE

Identification and characterization of the trehalose gene (*OtSA*) in some pathogenic bacteria using an *in silico* approach

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Abstract

Trehalose-6-phosphate synthase gene (*OtSA)* plays an important role in pathogenic interactions between plants and microorganisms. The genomic database confirmed the induction of several genes of trehalose involved in pathogenesis. The analysis of the available bacterial genome sequence database in GenBank confirmed the presence of trehalose-6-phosphate synthase gene *(OtSA)* for trehalose biosynthesis in some non-pathogenic bacteria. *E.coli, Pseudomonas* and *Salmonella* genomes are known to have the *OtSA* gene sequence in their genomes with accession numbers FJ895834.1, AY308798.1 and NC003198, respectively. In the present investigation, the conserved regions among these genomes were used to construct the degenerate primers that were used to amplify a 530 bp of *OtSA* related sequences in these non-pathogenic bacteria . In addition ,we used the *in silico* approach to investigate the existence of a natural sequence of trehalose (*OtSA*) gene in some pathogenic bacteria .i.e. *Pseudomonas aeruginosa* (ATCC 9027) ,*Bacillus cereus* (ATCC 33018) , *Pseudomonas luteola* API-20 NE using the same pairs of the degenerate primers . The fragments of these three genes were amplified by PCR, then sequenced and submitted into the GenBank /EMBL/DDBJ. These three sequences were accepted with the accession numbers : KJ808665, KJ808666 and KJ808667 for *Bacillus cereus, Pseudomonas luteola* and *Pseudomonas aeruginosa*, respectively.

Introduction

Trehalose (1-alpha-D-glucopyranosyl-1-alpha-D-glucopyranoside) is a non-reducing disaccharide which serves different biological roles in different organisms. In bacteria (*Escherichia coli* and *Bacillus subtilis*) it can be used as a carbon source, or synthesized as a compatible solute under osmotic-shock conditions (*E. coli*), or plays a structural role in *Corynebacteriaceae* as reported by Toms *et al.* (2010).

Pseudomonas aeruginosa is an opportunistic human bacterial pathogen that infects a wide range of plants including the model laboratory plant *Arabidopsis thaliana. P. aeruginosa* utilizes many of the same virulence-related factors to infect both plants and animals. However, because plants have fundamentally different cellular architecture than animals, it was hypothesized that *P. aeruginosa* synthesizes specific factors required for infecting plants but not animals. It was found that synthesis of the sugar molecule trehalose, an unusual dimer of glucose, is required for plant but not animal pathogenesis. Therefore, *P. aeruginosa* mutants defective in trehalose synthesis are non-pathogenic in *Arabidopsis thaliana. Arabidopsis* mutants that lack the polysaccharide xyloglucan in their cell walls can be infected by *P. aeruginosa* trehalose mutants (Slavica *et al*., 2013)

Initially, the role played by trehalose during the process of infection was deduced from studies where gene disruption of the trehalose-6-phosphate synthase (*TPS1*) gene in *Candida albicans* caused a decrease in the pathogenic infection, drastically affecting the development of hyphae (Zaragoza *et al.,* 1998).On the other hand, trehalose-6-

phosphate phosphatase (*TPS2*) gene knockout generated hyperacumulation of trehalose-6-phosphate phosphatase *(T6P)*, thermosensibility and cellular death in *Candida albicans* after a few hours of growth at 44 °C (Van Dijck *et al* ,2002).

Mutation of *OtSA gene* in the rice pathogen *Magnaporthe grisea*, caused a significant drop in plant infection apparently by inadequate penetration of the hyphae into the vegetative cells due to turgor loss .However, there is not yet a clear mechanism to explain the role of the trehalose in pathogenic interactions (Foster *et al*., 2003).

Trehalose is also known to be present in certain symbiotic interactions such as *endomycorrhizae* and *ectomycorrhizae* and in legume nodules (Brodmann, *et al*., 2002). Suarez *et al*. (2008) showed that the trehalose accumulated in *Rhizobia* during symbiosis with legumes. In this respect, a mutant form of the homologue gene of the *OtSA* of the *Rhizobium etli* resulted in overexpression of that gene. However, this line of experimentation was used to analyze their effects on the survival of free-living bacteria and the symbiotic process with *Phaseolus vulgaris*.

Trehalose can be synthesized *via* different metabolic pathways, sometimes coexisting, with both shared and specific functions relevant to pathogenicity. Of the three potential trehalose biosynthesis pathways differently contributing to *Mycobacterium tuberculosis* virulence, the *OtSAB* pathway is the dominant one. Deletion of *OtSA* results in severe growth defects, whereas the *OtSB* gene is essential for viability. Of the two additional pathways, the maltooligosyltrehalose synthase (*TreY*) and maltooligosyltrehalose hydrolase (*TreZ*) pathway, only the latter seems necessary for late development of chronic infections in mice, while absence of the first doesn't cause any apparent defect in microbial growth or virulence (Iturriaga *et al*. ,2009).

The genomic data revealed induction of several genes of trehalose involved in the pathogenesis process. With the aim of identifying the biosynthesis genes for trehalose in all completely sequenced genomes, a detailed analysis was undertaken using database searches for trehalose-6-phosphate phosphatase (*TPS*) and trehalose-6-phosphate synthase (*TPP*) sequences. It was discovered that eubacteria possess five routes for the biosynthesis of trehalose and archeae has four routes. On the other hand, the *OtSA* and *OtSB* domains in prokaryotes are generally found in their operons and exhibit similar DNA sequences to that of the *TPS* and *TPP* genes in eukaryotes. Whereas, in eukaryotes these domains have undergone parallel evolution as well as duplication (Avonce *et al*., 2006)

The use of the degenerate primers to amplify related genomic sequences or gene families for the determination of evolutionary relationships among homologous genes was tackled. In this respect, one can examine a single gene family among different organisms (orthologous genes) or examine several genes within the same organism (paralogous genes) (Rose, 2005).

The large number of identical and conserved residues present throughout the length of the alignment gives confidence that the observed alignment is reliable by using ClustalW algorithm to produce. The global alignment of trehalose sequences shows many regions that are nearly completely conserved in all species and degenerate primers based on these sequences have been successfully used to clone the gene encoding trehalose from a wide range of prokaryotic and eukaryotic organisms (Mohamed *et al* ., 2010).

In this study we demonstrate that the bioinformatics tools could used to design the degenerate primers based on a manual inspection of a multiple alignment of a small number of sequences of *OtSA* gene in non-pathogenic bacteria, i.e., *E.coli, P.savastanoi* and *S. enterica*. Regions of conservation could be determined by DNAStar Lasergene V7 software. Therefore, the degenerate primers were designed based on the regions of conservation. The program uses a multiple sequence alignment of amino acid sequences or DNA sequences as input. Then, the clustering techniques were mplemented to perform an automatic grouping of the input sequences based on the presence of the conserved regions for the members of the cluster .The *in silico* PCR module was performed and the correct melting temperature Tm was calculated and adjusted to be used in the thermal cycler. Finally, the designed degenerate primers were used to identify *OtSA* gene in the three studied pathogenic bacteria. The reason for choosing the pathogenicity as the focal point of this research study is the importance of the trehalose in the inhibition of pathogenicity pathway. For example, *Pseudomonas aeruginosa* strain PA14 is a multi-host pathogen that infects plants, nematodes, insects and vertebrates. Many PA14 factors are required for virulence in more than one of these hosts. In this respect, Slavica *et al*. (2013) sought to identify PA14 factors that are specifically required for plant pathogenesis. The author showed that synthesis by PA14 of the disaccharide trehalose is required for pathogenesis in *Arabidopsis*, but not in nematodes, insects, or mice. In-frame deletion of two closely-linked predicted trehalose biosynthetic operons, *treYZ* and *treS*, decreased growth in *Arabidopsis* leaves about 50 fold.

Material and Methods

Computer analysis of trehalose (*OtSA*) gene in non-pathogenic and pathogenic bacteria by Basic local alignment search tool (blast).

The DNA sequence of *E.coli* K-12 *OtSA* gene was retrieved from the sequence database of the National Center for Biotechnology Information (NCBI) with accession number AP012306.1 . The format of FASTA file was used as a query sequence to hit the database of GenBank and the category of database that was chosen was a non-redundant database and the algorithm type of the blast tool was blast nucleotide (blastn). This accession number was used to pick up related *OtSA* gene sequences in different non-pathogenic and pathogenic bacteria by blast tool. Then, we used the three accession numbers, (AP012306.1, of *Escherichia coli* K-12 , AY308798.1 of *Pseudomonas savastanoi* and NC003198 of *Salmonella enterica*) , which were retrieved from blastn since they gave the highest maximum identity. The score of an alignment was calculated as the sum of substitution and gap scores. Also, the significance of each alignment was computed as an Expectation value (E). E value means the number of different alignments with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E value, the more significant the score.

Determination of the conserved regions using the Mealign software tool

The Mealign module, which is implemented in the DNAStar Lasergene V7, was used to detect the consensus sequence among the three previously mentioned sequences for *OtSA* gene in non-pathogenic bacteria, i.e , *E.coli* K-12 , *P. savastanoi* and *S. enterica* . The clustalw multiple sequence alignment analysis was used to align more than one sequence and to produce the conserved blocks. These conserved blocks were employed design the degenerate primers in the Mealign module.

The Module of primer select of DNAStar Lasergene V7

The Module of primer select of DNAStar Lasergene V7 was used to locate the specific primers. The location of consensus sequence was assigned to be from 225 to 1297 bps and the product length was adjusted to be 300-580 bp. Then, all the parameters of the primer characteristics were adjusted, i.e., melting temperature (Tm), the primer length, hairpin loop, the overall stability and primer stability of the 3'end.

Checking the specificity and uniqueness of the designed primers.

Primer Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-Blast) was used to detect the homology between the designed selected primers from DNAStar Lasergene V7 and the targeted templates in the GenBank database. Moreover, the position of the degenerate nucleotides and the uniqueness for the forward and reverse primers were assessed. Also, the PCR *in silico* Module was empolyed predict and to produce all the true condition of PCR program. Finally, the degenerate primers were designed based on DNA computer analysis and the *in silico* PCR approach.

Bacteria strains and growth conditions

The bacteria strains, *i.e.., E. coli* (ATCC 10538*), B.subtilis* (ATCC 6051) *P.aeruginosa* (ATCC 9027) and *B. cereus* (ATCC 33018) were kindly provided by the Microbiological Resources Center (MIRCEN), Faculty of Agriculture , Ain Shams University. While *P. luteola* (API-20 NE) was provided by the Microbiological Center, Faculty of Agriculture, Cairo University. These bacteria were inoculated into LB medium containing 10g/L Tryptone,10 g/L NaCl , 5 g/L Yeast Extract, pH 7 and incubated in a rotary shaker at (37 ℃ for *E.coli* and 30 ℃ for *B. subtlis* , *P. aeruginosa* ,*B. cereus* and *P. luteola*) and 150 rpm for 24 h. Thenafter, 1.5 ml culture broth was centrifuged at 5000 rpm for 10 min to collect cells.

Genomic DNA isolation and PCR amplification technique

Genomic DNA was extracted from the collected bacterial cells according to Karakousis *et al*. (2006). Two sets of degenerate primers were used for the detection of the *OtSA* gene sequence in the genome of the pathogenic and non-pathogenic bacteria by the PCR technique. Different conditions and annealing temperatures for every bacterial

species were tried through the course of our experimentation. The PCR reactions was carried out in a 20 µl volume containing 2 µl of DNA (50-100 ng/ µl), 1 µl for each primer (10 pmole /1ml primers , primers were synthesized at Metabion International AG), 10 µl Master Mix of thermostable DNA polymerase (Jena bio-company) and 6 µL ddH2O). The PCR was carried out using a DNA thermal cycler (Techne -312 andBiometra-unoII). The PCR conditions were as follows : 94 °C / 3 min, then 40 rounds of 94 °C / 60 sec, Tm was 50 °C , 57 °C and 58 °C for *B. cereus, P. aeruginosa* and *P. luteola*, respectively, 72 °C 1/ min followed by 72 °C 10 minutes). The above mentioned PCR condition was also applied for the non-pathogenic bacteria but with different Tm, i.e, 50 °C for *E. coli* and 56 °C for *B. subtilis*. The PCR products were electrophorezed on 1% agarose gel. Finally, the three PCR products for the *OtSA* of *P. aeruginosa* , *B.cereus* and *P. luteola* were cut from the gel and purified according to Q gene band elution kit.

DNA sequence analysis of the amplified products of the studied pathogenic bacteria.

Purified PCR product for the expected trehalose gene *(OtSA)* of the three studied pathogenic bacteria, *i.e., P. aeruginosa, B. cereus* and *P. luteola* were sent to Microgen American company for sequencing. Then, verification of the amplified sequence in comparison to those available in the GenBank was carried out. The consensus sequences of the three studied pathogenic bacteria sequences were obtained by DNA Baser project software. Finally, these consensus sequences of the three studied pathogenic bacteria sequences were submitted to GenBank.

Submitting the three partial sequences of *OtSA gene* of the studied pathogenic bacteria to GenBank using bankit method.

The three partial sequences of the *OtSA* gene of the three studied pathogenic bacteria were submitted, processed and accepted. The bankit number 4582222 is shown in the GenBank flat file. Based on the submitted data to the GenBank, the schedule release date for the submission was Aug 25, 2014.

Results and Discussion

The use of degenerate primers on complex samples may allow the determination of the diversity of sequences of the gene family within the sample or the identification and characterization of unknown, related members of the gene family (Rose, 2005). Homologous genes typically contain a mix of highly conserved regions and divergent regions. Evolutionary distances are determined from the nucleotide or amino acid differences within the divergent regions, while primers are created from the highly conserved regions flanking a divergent region. The ideal primer for a particular protein or gene family would amplify every member of the group within a sample and nothing more. With the aim of identifying the biosynthesis genes for trehalose in all completely sequenced genomes, a detailed analysis was undertaken using database searches for *TPS* and *TPP* sequences. It was discovered that eubacteria possess five routes for the biosynthesis of trehalose and archeae has four routes, lacking the TS pathway. Fungi, plants and metazoa only possess the TPS/TPP pathway. Interestingly, among vertebrates there are no genes for the biosynthesis of trehalose, but the trehalase gene is present. On the other hand, the *OtSA* and *OtSB* domains in prokaryotes are generally found in operons, whereas in eukaryotes these domains have undergone parallel evolution as well as duplication (Avonce *et al*., 2006).

The use of bioinformatics analysis to search for (*OtSA*) related gene (s) in pathogenic bacteria.

The nucleotide sequences of trehalose-6-phosphate synthase (*OtSA*) gene from different non-pathogenic and some pathogenic bacteria were retrieved and saved as sequences in FastA format and each gene was copied and pasted into a new separate file. This FastA list of sequences was used to perform the nucleotide-based multiple sequence alignment. The blast program was used to determine the different sequences of *OtSA* gene in different bacteria .The analysis of the available bacteria genome sequence database in GenBank led to that most of the non-pathogenic bacteria have *OtSA* sequences in their genome. These sequences of the *OtSA* gene were not detected in some pathogenic bacteria i.e. *Bacillus cereus, Pseudomonas luteola* and *Pseudomonas aeruginosa.* However, the presence of *OtSA* gene sequence in the pathogenic bacterial genome was determined in this study using the conserved regions of the *OtSA gene* sequences in the different non-pathogenic bacteria.

Retrieving the conserved sequences of the *OtSA* gene in the non-pathogenic bacteria by the blast analysis

The blast program produced a local alignment between the query sequence AP012306.1 of *E.coli* K-12 and the other strain records in the database i.e. AY308798.1 for *P.savastanoi* and NC003198 for *S. enterica*. Each pairwise local alignment exhibited a bit score (normalized score of the alignment) and the optimal score of alignment (OPS). The distance of similarity between the query sequence and the targeted sequences shows a significant value or otherwise. That depending on the E-value for every bit scores of pairwise alignment. However, the quality of the alignment is judged or evaluated by the value of score (S) and the E value (E). The E-value reflected the significance of similarity between the query sequence and the targeted or subjected sequences as shown in Table (1). Table (1) summarizes the homology results between the query sequence of accession number AP012306.1 of *E. coli* and the subjected records in the database. The subjected selected records were AY308798.1 for *P.savastanoi* and NC_003198 for *S.enterica*. These two records which gave the highest maximum identity (99% and 98%, respectively) were homologous to query sequence that have the maximum identity of 100 % . Moreover, all the above mentioned sequences were selected depending on their expected values (E-values) which were equal to zero for all the three previously mentioned non-pathogenic bacteria. Finally, the maximum score for *E. coli*, *P.savastanoi* and *S.enterica* were 2632, 2621 and 1086, respectively. These sequences were retrieved by BLASTn program depending on the highest score of the pairwise alignment for every accession number and the significance of these scores were measured by the E-value. In addition, it was determined , which parts are conserved or variable sequences. Based on these information, the conserved DNA motifs were used as the starting points to design the degenerate PCR primers.

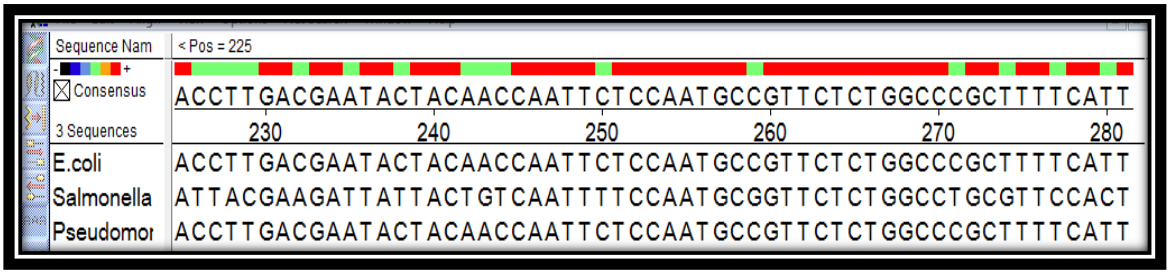
Table 1: Accession number of sequences of the different non-pathogenic bacteria producing the significant alignments.

Accession No.	Description	Max score	E value	Max identity
AP012306.1	*Escherichia coli* K-12 (*OtSA*)	2632	0.0	100%
AY308798.1	*Pseudomonas savastanoi* BCNU 106 (tps)	2621	0.0	99%
NC_003198	*Salmonella enterica* CT18 (*OtSA*)	1086	0.0	98%

Determination of the conserved regions among the analyzed sequences in some non- pathogenic bacteria

The *OtSA* sequences of the three previously mentioned non-pathogenic bacteria were saved using EditSeq in Lasergene. DNA sequence format was opened by Mealign tool of the DNAStar Lasergene to generate the multiple sequence alignment among these three sequences through ClustalW algorithm. After completing the multiple alignments, variant degrees of conservation were produced and then the highly conserved blocks (red bars) were determined. Therefore, the red bars of the histogram showed the best positions of the consensus sequences spanning from the nucleotide position 225 to 1297 bps as shown in Fig.(1). The few degenerate nucleotides were visualized as green segments in the consensus bar (Fig.1).Thereforer , these consensus sequences appeared in the position from 225 to 1297 bps and that range was used to produce the degenerate primers pair. The above mentioned approach was also applied to a number of different bacteria species. This approach failed to produce the conserved regions with few degeneracy nucleotides. In this respect, Shelef (2007) pointed out that organisms with similar codon biases may be more conserved at the DNA nucleotide level than would be expected using amino acid degeneracy alone

Part 1



Part 2

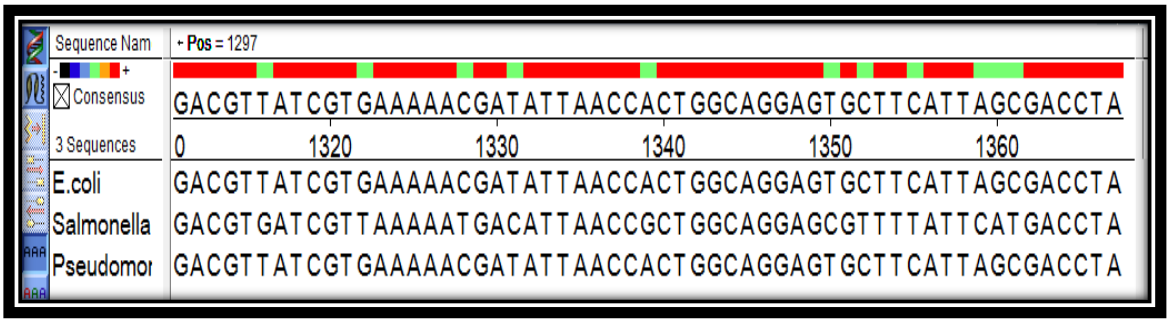


Fig. 1.The conserved sequences presented in two parts (part 1 and part 2) were produced by multiple sequences of *E.coli, Salmonella,* and *Pseudomonas*. The consensus sequence (+) appeared as red bars beginning from position 225 to 1297 bps.

Identification of the selected primers using the Primer Select tool

The previously mentioned consensus sequence was used to produce the forward and the reverse primers using the PrimerSelect module of DNAStar Lasergene. This module was used to locate the specific primers .The consensus sequence was assigned to be from 225 to 1297 bps and the product length was adjusted to be 300-550 bps. However, the length of the expected product was 530 bps in length. The module of PrimerSelect was also used to produce the report of the amplification summary as seen in Fig.(2). The information about the forward primer 5'AAG GTTTGCCAGAGCGTTTTC 3' and the reverse primer 5' TGCCAGTGGTTAATA TCGTTTTTC3' like the melting temperature (Tm) are described and documented in in Fig. 2. The (Tm) of the forward primer was equal to 55.1 ℃ while the (Tm) of the reverse primer was 54.3 ℃ . The primer Tm difference was equal to 0.8 ℃ . The small difference of the Tm indicates the best primer design parameters.

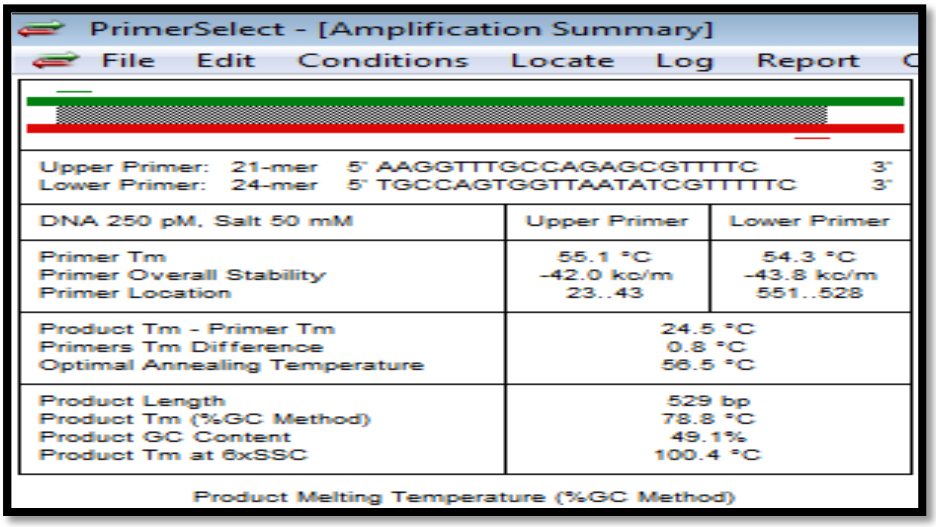


Fig. 2. The amplification summary showing the melting temperature (Tm) for forward and reverse primers, primer stability on the template of DNA and the primer location.

Checking the quality and uniqueness of the designed primers and defining the degeneracy of the nucleotide in the DNA sequence.

The primer pair was aligned by the primer Blast tool. This tool was used to check the specificity of the selected primers and to detect the homology between the subject records of the *OtSA* gene and those of the two primers. The results revealed that the best alignment of the forward and the reverse primers was with the *E. coli* K-12, *P. savastanoi* and other non-pathogenic bacteria, i.e. , *Shigella sonnei* and *Enterobacter asburiae* had different degenerate sequences. Moreover, the mismatched bases between the primer and the templates indicated the degenerate bases as presented in Table (2). The results indicated that the designed primers are the best because the conserved regions effectively produced highly degenerate and then highly specific primers for 39 non-pathogenic bacteria. However, the sequences of this gene with known regions of high variability are recorded in the GenBank as *OtSA* gene. Some of these data are manifested in Table (2). The degenerate nucleotide sequences of the designed forward primer for *E. coli*, *P.savastanoi* , S. *sonnei* and *E. asburiae* were (G,A, G) while in reverse primer they were (G,G,A) in *E. asburiae*. Another advantage of the DNA alignment-based primers is the length of the PCR product covering the 530 bps regions. Degeneracy in the primers is required for all possible variants of a gene family to be amplified and compared (Shelef, 2007)

These degenerate sequences were used to design the degenerate primer pair as summarized in Table (3). The degenerate primers can then be designed where Y(C,T)- N (A,G,C,T) -V (A,G,C)-D (A,G,T) R(A,G)-B(G,C,T),M(C,A),W(A,T). Shelef (2007) used a different and unique approach to design the degenerate primers where all comparisons can be made. For example, all of the programs use a multiple sequence alignment of the researcher's sequences of interest as input. However, some of the programs (CODEHOP) use an amino acid-based multiple sequence alignment. While others (HYDEN, Primaclade, Greene SCPrimer) use a DNA nucleotide-based multiple sequence alignment. The DePiCt program is a unique one as it can accept either types.

Table 2: Homology analysis between the designed primer and the subjected records of the *OtSA gene* in the database using the primer blast tool.

Template or species	**Degenerate nucleotides in designed primer** **R primer** **F primer**	
≥AP009048.1 *E. coli* str. K12, for *OtSA gene* have product length 530 **≥AY308798.1 *P.savastanoi* str. BCNU 106, for *TPS* gene product length 530** **>CP000038.1 *S. sonnei* str.Ss046, for *OtSA gene* product length 530** >**CP003026.1 *E. asburiae* str.LF7a, for *OtSA gene* product length 530**	Forward primer 1 Template 1982948 AAGGCTTTGCCAGAGCGTTTTC 22 ..A.G.....1982927	Reverse primer 1 Template 1982419 TGCCAGTGGTTAATATCGTTTTTC 24 1982442
	Forward primer 1 Template 802 AAGGCTTTGCCAGAGCGTTTTC 22 ..A.G..... 823	Reverse primer 1 Template 1331 TGCCAGTGGTTAATATCGTTTTTC 24 1308
	Forward primer 1 Template 1296538 AAGGCTTTGCCAGAGCGTTTTC 22 ..A.G.....1296559	Reverse primer 1 Template 1297067 TGCCAGTGGTTAATATCGTTTTTC 24 1297044
	Forward primer 1 Template 2805705 AAGGCTTTGCCAGAGCGTTTTC 22 ..A.G.....G.....2805684	Reverse primer 1 Template 2805176 TGCCAGTGGTTAATATCGTTTTTC 24 .....G..G..A.....A280519

Table. 3 : The nucleotide sequence of the designed forward and reverse degenerate primers.

Primer	Nucleotide sequences 5' to 3	Expected fragment (bp)
Forward primer	5' F'AAGGKYTGCCRGARCGTTTTC 3'	530
Reverse primer	5'TGCCAGYGGTTRATRTCRTTTTTV GC3	530

Testing the degenerate primers pairs on some of non-pathogenic bacteria using PCR *In silico* approach.

One important application of the degenerate primer design is to explore and investigate the evolutionary relationships and diversity within a functionally important gene family. The designed degenerate primers were first tested for detecting the *OtSA* gene sequence in organisms that are naturally known to have this gene, i.e., *P. savastanoi*, *E. coli K12*, Shigella *dysenteriae* and *S. sonnei*. The expected PCR product was electrophoretically resolved at 530 bps in these organisms using the *in silico* PCR approach as presented in Table (4).

The *in silico* PCR module of the FAST PCR program was very attractive and quick for analyzing primers through target template sequences, determination of primer location, orientation, efficiency of binding, complementarity and Tm calculation. The *in silico* PCR module could detect the percentage of similarity between the designed primer pairs and the target template sequences. Table (4) shows the similarity scores, the true positions of the aligned primers with the template sequences and the Tm dimer for forward and the reverse primers for every studied bacteria, i.e., *P. savastanoi*, *E. coli K12*, S. *dysenteriae* and *S. sonnei* .The results revealed that the *in silico* PCR Module reflected the predicted homology between the *OtSA gene* sequence in different non-pathogenic bacteria and that of the designed degenerate primers in lab. Therefore, to confirm these results PCR amplification was applied for the non-pathogenic bacteria as shown in Fig.(3). The PCR products for *E.coli* and *B. subtilis* were detected at MW of 530 bp using the two sets of degenerate primers. These products were related to the consensus coding region of the *OtSA gene* family. In conclusion, these two bacterial species are sharing the homologous consensus region of the trehalose gene (*OtSA*) with the other members of the gene family.

Table.4. *In silico* PCR product prediction

Species and Similarity	Complementary relationship between primer and template	Tm dimer C°	Aligned position
*P.savastani*o (73%)	<-vtttttrctrtarttggygaccgtcttttgcragrccgtykggaa- :|||||:||||||||:|||||||:|: ||| :: ::| | Gtgaaaaacgatattaaccactggcaggagtgcttcattagcgacctaaag	50.7°	1308<-1355
E. coli .K12 (69%)	<-vtttttrctrtarttggygaccgtcttttgcragrccgtykggaa-5 :||||:||||||||:|||||||:|: : ||| :: ::| | gtgaaaaacgatattaaccactggcaggagtgcttcattagcgacctaaag	51.3°	1308<-1352
S. dysenteriae (69%)	>aaggkytgccrgarcgttttcttctgccagyggttratrtcrttttv-5 ::: |: | :| : : ||:|||||||:|||||||||:|||||: Gctttaggtcgctaatgaagcactcctgccagtggttaatatcgtttttcac	48.0°	71->118
S.sonne (70%)	<-vtttttrctrtarttggygaccgtcttttgcragrccgtykggaa-5 :||||:||||||||:|||||||:|: : ||| :: ::| | gtgaaaaacgatattaaccactggcaggagtgcttcattagcgacctaaag	49.0°	1308<-1352

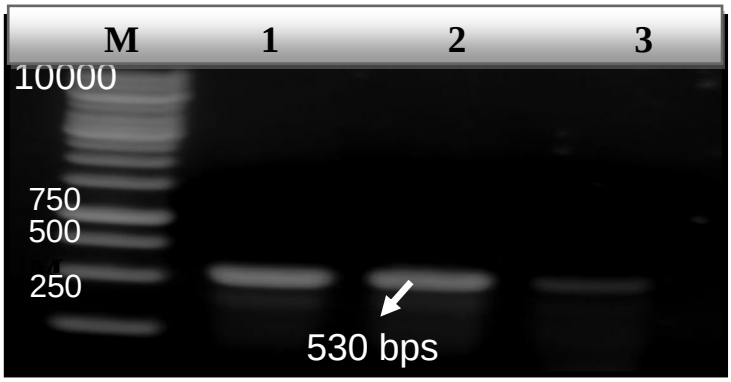


Fig.4. PCR amplification using the degenerate primers pair to detect the similar consensus region in trehalose gene for non-pathogenic bacteria. 1 and 2 :*E.coli* ; 3: *B. subtilis.*; M: 1Kb DNA ladder

PCR amplification for the pathogenic bacteria to detect the PCR product using the designed degenerate primers.

Another important application of the degenerate primers design is the identification and characterization of the unknown and related members of the gene family (Rose, 2005) .Therefore, the two degenerate primers were used for amplifying the expected PCR product (530 bp) in the three pathogenic bacteria *P. aeruginosa*, *B. cereus* and *P. luteola*, as shown in Fig.(5).

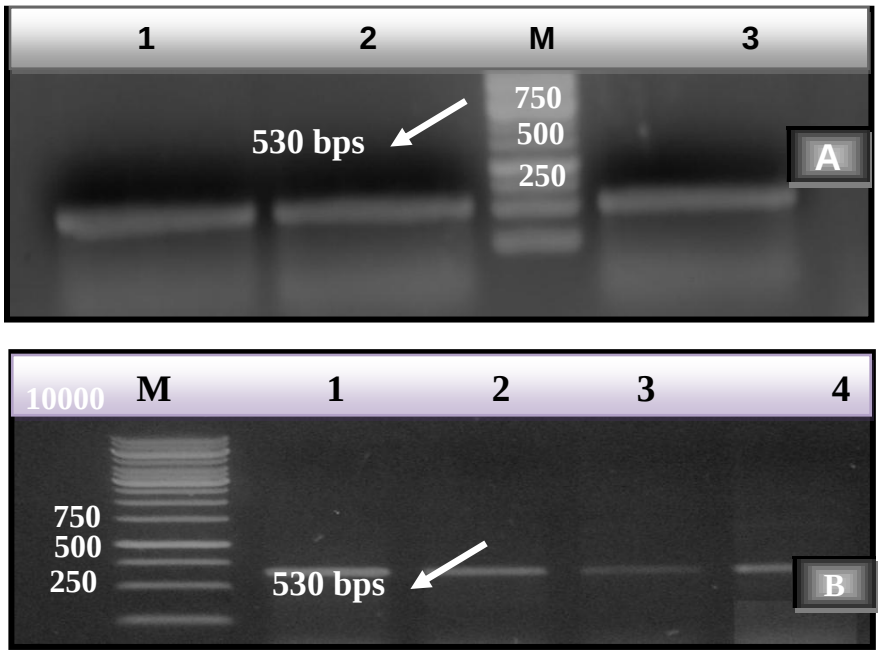


Fig.5. Amplification of the PCR product at MW 530 bp , using the degenerate primers pair to detect the *OtSA* gene. A : unpurified ; B : purified . 1: *P. luteola ;* 2: *B. cereus ;* 3 and 4 : *P. aergenoza ;* M: 1Kb DNA ladder .

DNA sequence analysis and verification of the three amplified sequence for studied pathogenic bacteria in comparison to those available in the GenBank.

The results of electropherogram using direct sequencing PCR product for the forward and reverse degenerate primers (sharp and high peaks) until 530 bp in the three pathogenic bacteria ,.i.e ,*Bacillus cereus*, *Pseudomonas luteola* and *Pseudomonas aeruginosa* were analyzed. The sequencing data were exported to DNA baser assembler to produce and determine consensus region between the two pairs of degenerate primers in the three pathogenic bacteria.

After DNA sequencing the results revealed that the trehalose-6-phosphate synthase (*OtSA*) gene fragments are identified in the three studied pathogenic bacteria .The nucleotide sequence of the three gene fragments was submitted into the Genbank / EMBL/DDB and given the accession numbers KJ808665, KJ808666 and KJ808667 for *Bacillus cereus*, *Pseudomonas luteola* and *Pseudomonas aeruginosa.*, respectively.

The results presented here are the first study to use the *in silico* approach to investigate the existence of the natural sequence of the trehalose gene (*OtSA*) in the three studied pathogenic bacteria.

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