CLONING AND EXPRESSION OF GENES ENCODING ENZYMES INVOLVED IN THE SYNTHESIS OF PHENOLIC ANTIBIOTIC IN RECOMBINANT E. COLI

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ABSTRACT
Methicillin-resistant Staphylococcus aureus (MRSA) causes serious public health problems throughout the world. In order to find an appropriate antibiotic against S. aureus scientists have focused on 2,4-diacylphloroglucinol (DAPG) antibiotic produced by certain Pseudomonas spp. strains. Pseudomonas sp. strains residing in the rhizosphere have been considered due to their ability to produce antimicrobial metabolites which protect plants against different types of pathogens. In this study, Pseudomonas fluorescens UTPf100 was selected among 103 different Iranian isolates as an overproducer of DAPG on the basis of biological control and DAPG production. From this strain we isolated four genes that directly affect DAPG biosynthesis. The isolation was then confirmed by nested PCR and digestion. The encoding sequences responsible for DAPG biosynthesis were sub-cloned to pET28a (+) as an expression vector and transformed in E. coli BL21. To the best of our knowledge this is the first report for expression of the operon responsible for DAPG synthesis in recombinant E. coli.


Keywords: biological control, phenolic antibiotic, Pseudomonas, expression

Abbreviations:
DAPG: 2, 4-diacylphloroglucinol;
IPTG: isopropyl-beta-D-thiogalactopyranoside;
X-gal: bromo-chloro-indolyl-galactopyranoside;
MRSA: methicillin-resistant Staphylococcus aureus

Introduction
Although 2,4-diacetylphloroglucinol (DAPG) is known to be produced by Pseudomonas sp. and is an antifungal and antibacterial substance, there are not many reports about its medical application. Methicillin-resistant Staphylococcus aureus (MRSA) causes serious common health problems in the world. Isansetyo et al. (8) screened various species of marine alga and identified several alga species that showed anti-MRSA activity. Eventually Pseudomonas sp. AMSN isolated from the surface of these alga species proved to produce an anti-MRSA substance. This effective substance became known as DAPG. DAPG, among the commercial antibiotics tested, was shown to have the strongest bactericidal activity (8). Despite the fact that genes for resistance to foliar pathogens are abundant in most plant species, there are not many examples of resistance to a large part of the most common and widespread soil-borne pathogens (Geumannomyces graminis, Pythium sp., Rhizoctonia spp., Fusarium spp.) (16, 17). In this respect, DAPG was introduced as a most effective compound for the protection of plants against pathogens. As a solution, plants have adopted a support and stimulation strategy towards a specific group of pathogen antagonists of thousands of species of beneficial and harmful pathogens in the rhizosphere environment as a natural defense system against soil-borne pathogens (17). These diseases cause much damage in crops and garden produce. The use of bacterial and fungal bio-control agents is a promising control method for soil-borne pathogens (14). One of the most effective and important mechanisms used by bacterial and fungal agents is the production of antimicrobial compounds such as antibiotics (16). On the other hand, among the most important group of plant resistance factors and root defense against soil-borne pathogens, antibiotic producing pseudomonads play a key role (10, 14). One of these metabolites is the DAPG antibiotic produced by a wide range of Pseudomonas sp. This antibiotic has been highly considered by researchers in root disease (11, 16). DAPG is a phenolic compound with a broad spectrum of anti-fungal, anti-bacterial, anti-viral, anti-nematode and anti-worm activity (7, 10, 11, 15). The genes that give antibiotic-producing strains the ability of production are on the 6.5 kb fragment in the P. fluorescens Q2-87 genome. Nucleotide sequence analysis of this region has revealed six genes in three transcription units, including four genes that constitute the phiACBD operon. Studies have shown that all four gene protein products are needed for both monoacetylphloroglucinol (precursor) and 2,4-diacylphloroglucinol synthesis. The phiACBD operon is adjacent to two other genes that are separately transcribed. These two genes are known as phlE and phlF and code for a DAPG export protein and a regulator (repressor) respectively. They are not necessary for the synthesis of phloroglucinol (1).

It should be mentioned that, despite extensive research, only one percent of the agricultural pesticides include biological agents and only few biological pesticides contain Pseudomonas fluorescens. Most of this variation is related to the differences in the physical and chemical characteristics of the natural environments where the biocontrol agents are applied (5). This evidence shows the importance and necessity for characterization and use of new and efficient technologies for its production. Thus, the aim of the present study was to isolate DAPG genes from Pseudomonas fluorescens UTPf...
which has high antagonistic potential, and investigation of their expression in recombinant E. coli.

Materials and Methods

Organisms

*Pseudomonas fluorescens UTPF 100* isolate was obtained from Tehran University Plant Protection Department and *E. coli* (strain and its expression vector), from the Agricultural Biotechnology Research Institute of Iran, Gene Transfer Division.

Enzymes and chemicals

Polymerization and restriction enzymes, dNTP, antibiotic and ligation kit (Fermentas), chemicals (Merck), and pGEMT-easy vector System II (Promega) were used.

Primer design

Isolation of the DAPG operon from the genome of *UTPF100* was done by PCR. Primers were designed by OligoTech software based on the published sequence of Q2-87 (1). Test isolation was designed using nested PCR and enzyme digestion, so two primers were designed by Brian et al. (3) based on the PhlD gene from the nested PCR reaction.

Polymerase chain reaction

PCR was carried out in a 20 ml reaction mixture containing 25 ng of total DNA as a template, 0.8 mM primer, 3 mM MgSO4, 1.0 mM dNTP, 1× Pfx Buffer and 3U Pfx Polymerase enzyme. Amplification was performed in a Master cycler in 0.2 ml tubes. The PCR reaction program consisted of: 1 cycle at 95 °C, 5 min; 36 cycles of 93 °C, 1 min; 58 °C, 1 min; 68 °C, 5 min; 1 cycle 68 °C, 10 min.

Nested PCR

In this method the first PCR product and inner *phlD* primers supplement the second PCR reaction. A fragment of 629 bp length was expected if the fragment isolated in the first PCR was synthesized correctly.

Recovery and cloning

We used High Pure PCR Product Purification Kit for recovery and purification of the PCR product after running it on the agarose gel. Competent cells were prepared according to the standard protocol by low temperature growing and CaCl2 salt (12). The *PhlACBD* operon was purified in the pGEMT-easy vector system I cloned by the manufacturing company’s protocol. This vector is linear because it has a T nucleotide at the 3’ end, so we added an A nucleotide to the 3’ end of the operon according to the manufacturer’s protocol. The restriction mixture was kept at room temperature for 1 hour and to enhance the ligation efficiency it was incubated overnight at 4 °C. Then it was transformed to *E. coli* XL1-blue by thermal shock (12) and 100 ml of putative transformed bacteria were spread on LB selective media containing ampicillin antibiotic, IPTG and X-Gal, and incubated at 37 °C for 24 h, then some colonies were selected and cultured for cloning testing.

Cloning confirmation

After plasmid isolation (4) from the colonies formed, PCR and digestion were applied for cloning confirmation. PCR was performed with internal primer and *Taq* enzyme. The digestion was performed with *NotI* and *EcoRI* restriction enzymes. These enzymes do not have any restriction sites within the insert. After the restriction reaction two fragments are expected with lengths of 4 kb (vector) and 4 kb (insert).

Sub-cloning to expression vector

Both vector and cloned insert were cut with *EcoRI* enzyme, after treating the expression vector with alkaline phosphatase enzyme, ligation reaction was performed using the T4 DNA Ligation Kit according to the manufacturer’s instructions. Then we transformed the recombinant plasmid to XL1-blue strain, after investigating the insert’s position and confirming the correct position, the plasmid was isolated and transformed to strain *BL21*. Strain *BL21* has an RNA polymerase with the ability to recognize the T7 promoter and to transcribe the cloned insert.

Investigation of insert position

For correct transcription of the sense strand, the 3’ end of the insert should ligate to the 5’ end of the promoter. To investigate the insert’s position, *BamHI* enzyme was used with a restriction site in the vector and another one in the insert. The plasmid from 20 colonies formed on selective media (containing kanamycin) was obtained by alkaline lysis (3).

Induction of promoter

Insertion in pET vector is under T7 promoter and RNA polymerase for identification of T7 promoter regulator Lac operon; so IPTG was used for induction of the promoter. IPTG was added in four different concentrations: 0.2, 0.3, 0.4, and 0.5 mM, according to the manufacturer’s protocol.

SDS-PAGE analysis

Protein was estimated by the Bradford method (2). SDS-PAGE was done according to the Laemmli method. Gels were stained with Coomassie Brilliant Blue R-250. To investigate the effects of different carbon resources and different media on the recombinant antibiotic expression. Glucose and sucrose were

<table>
<thead>
<tr>
<th>bp</th>
<th>Nucleotide sequence</th>
<th>Primer</th>
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<tr>
<td>18</td>
<td>5’- GGAATCTGGAGGAAGTAC -3’</td>
<td>DAPG-F</td>
</tr>
<tr>
<td>18</td>
<td>5’- ATGTTTGGTCGTCGGGAA -3’</td>
<td>DAPG-R</td>
</tr>
<tr>
<td>25</td>
<td>5’- ACCACCCGGCGACATCGTTATGAGC -3’</td>
<td>DAPG-I phlD F</td>
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<tr>
<td>25</td>
<td>5’- CGCCGTATGGAAGATGAAAAGTC-3’</td>
<td>DAPG-I phlD R</td>
</tr>
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List of the primers used in this study
added to LB and KingB media and expression was investigated by SDS-PAGE.

Results and Discussion
The PCR technique was performed with the Platinum Pfx enzyme and the specific primer was annealed under different temperatures. A 4-kb band appeared on the 1% agarose gel when 58 °C annealing temperature was used (Fig. 1). In order to prove that the DAPG operon was isolated and identified properly nested PCR was used. A smaller part of the isolated fragment was selected and the primer was designed based on it. The PCR product was used as a template in the second PCR. Considering the importance of the phlD gene for the synthesis of DAPG antibiotic and its presence in *Pseudomonas* bacteria identified in all environments, in this study part of the phlD gene was used to design the primer (3). If this isolation had been proper, we expected to observe a 629 bp band. The results in Fig. 2 show that the isolation was done properly. The isolated fragment was recovered by high Pure Product PCR purification kit, and then the mixture was transferred to pGEMT-easy vector at a 3:1 ratio and transformed to *E. coli* X-L1blue strain. Some white colonies as a putative clone were selected and tested by colony PCR and digestion (Fig. 3 and Fig. 4). After sub-cloning the fragment to pET28a (+), IPTG was added to the bacteria culture according to Novagen’s protocol for promoter induction. There was no bacterial growth at 0.3, 0.4 and 0.5 mM of IPTG (Fig. 5), which shows the toxicity of the DAPG antibiotic to the host cell (*E. coli*) at these levels of expression. It could be suggested that the expression and production of DAPG at these IPTG concentrations was high enough to be toxic. At 0.2 mM of IPTG, however, bacteria grew and the media became turbid (Fig. 5). This result could be interpreted in two possible ways. Either the genes encoding the DAPG antibiotic synthesizing enzymes were not expressed at all, or they were not expressed at high enough levels to be fatal for the host.

SDS-PAGE was used for investigation of proteins expression in the recombinant host cells. For this purpose proteins were extracted from induced bacteria, from transformed but non-induced bacteria, and from non-transformed bacteria. Then these samples were loaded on 10% SDS-PAGE, and run for 7 hours at 200 V. As shown in Fig. 6, induced bacteria had four additional bands compared to non-induced and non-transformed ones (the results for the non-transformed bacteria are not shown in the figure). The sizes of these additional bands were equivalent to the protein bands from the genes encoding the DAPG synthesizing enzymes as described by Bangera and Thomashow (1). This allowed us to conclude that the recombinant genes were expressed and antibiotic was produced.

Shanahan et al. (13) were the first to succeed in extracting and identifying DAPG antibiotic from *P. fluorescens*. After extensive research DAPG and other metabolites from different *Pseudomonas* species were isolated. Jamali et al. (9) investigated 93 strains of *P. fluorescens* and succeeded in identifying 9 strains which possessed the *PhlD* gene, the essential gene for biosynthesis of DAPG. Among them strain UTPF 100 had the most pronounced antagonistic properties, even more than those of global strains like CHA0 and Q2-87 (9). This property probably was due to the high production of antibiotics like DAPG. So UTPF 100 was selected and overexpressed in the recombinant host, but in the case of high expression it was detrimental to *E. coli*. We suggest using *Pseudomonas* engineered vector and transformation to *Pseudomonas* without antagonistic properties. It could be that *Pseudomonas* spp. are resistant to DAPG and in the case of overexpression the bacteria survive. When different media such as LB, NB and King B were used, no changes in the expression of the recombinant were seen. In all these media the specific protein bands of DAPG antibiotic synthesizing enzymes were observed. When glucose or sucrose was used as a carbon source, no changes were observed in the expression of the genes and the production of antibiotic.
Bangera and Thomashow (1) have separately expressed the genes which encode DAPG synthesizing enzymes in recombinant *E. coli* to study the structure of the enzymes, but, to the best of our knowledge, our study is the first report for expression of these genes in the form of an operon. These results are consistent with the findings of other researchers (13, 16) and this could be referred to as a new cassette of genes encoding DAPG.

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**REFERENCES**