Sequence Analysis of Novel Genes in Clinical and Environmental Pseudomonas aeruginosa Iraqi Isolates

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Study the sequencing of phzM and phzS genes and the effect of their mutation on phenazine production. Pseudomonas aeruginosa has the ability to adapt and grow in a variety of environments and is owing to its wide genetic flexibility. This organism produces a diverse group of phenazine including pyocyanin, phenazine-1-carboxylic acid and phenazine-1-carboxamide. The Phenazine-1-carboxylic acid that converted to pyocyanin is mediated by phzM and phzS novel phenazine modifying genes. The two genes that convert the phenazine-1-carboxylic acid to pyocyanin encode the putative enzymes, methyltransferase and monooxygenase, respectively, however, the overexpression of gene(s) encoding biosynthesis of pyocyanin via increasing the copy number has not so far identified in P. aeruginosa. Molecular technique was used to detect phenazine modifying genes (phzM, phzS), detection of mutations in these genes by sequencing. The results revealed that the phzM and phzS gene were present in 21(80.76%), 14(53.84%) respectively. Sequencing of phzM and phzS from isolates of P. aeruginosa reveal that 9 mutations in 8 isolates (7), (2) mutations for both genes respectively with the identities (99%) with the standard strand in NCBI web site.

Keywords: Phenazine modifying genes, P. aeruginosa, sequencing.

Pseudomonas is classified as a diverse genus of gram-negative aerobic bacilli of Gammaproteobacteria, belonging to the family Pseudomonadaceae. This bacterium is with more than 60 species displaying diverse lifestyles in various environments. Pseudomonas aeruginosa has the ability to adapt and grow in a variety of environments and is owing to wide genetic flexibility. It is a common source of hospital-acquired infections, mainly burned victims or immunocompromised hosts. Using the whole-genome sequence of P. aeruginosa, chromosomal alterations have been investigated between clinical and environmental isolates of this microorganism and have postulated that variable regions some of which encode virulence genes are not preserved between clinical isolates. It is a marine and soil organism and an opportunistic human pathogen able to infect different sites and tissues. Although the organism is especially distinguished for the pathogenicity, the molecular studies have directed light into the distinctive feature of this bacterium to produce different secondary metabolites with the many biotechnological applications. This organism produces a diverse group of phenazine including pyocyanin, phenazine-1-carboxylic acid and phenazine-1-carboxamide. The chloroform-soluble, blue-green phenazine pigment of P. aeruginosa has been recognized as antibiotic of wide-spectrum. The biosynthetic genes of phenazine are organized in one operon, phz ABCDEFG in all Pseudomonas species with the exception of Paeruginosa which keeps dual copies of these “core” genes responsible for biosynthesis of phenazine -1-carboxylic acid synthesis. The phenazine-1-carboxylic acid that converted to pyocyanin is mediated by phzM and phzS novel genes.
Table 1. Type of mutations in the \textit{phzM} gene sequence of \textit{Pseudomonas aeruginosa} in 4 isolates

<table>
<thead>
<tr>
<th>No. Of sample</th>
<th>Wild-type</th>
<th>Mutant type</th>
<th>Type of mutation</th>
<th>Location</th>
<th>Amino acid change</th>
<th>effect</th>
<th>Sequence ID</th>
<th>Identities</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>GGC</td>
<td>GGA</td>
<td>Transversion</td>
<td>784321</td>
<td>Glycine &gt; Glycine</td>
<td>nonsense</td>
<td>ID: CP020603.1</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>GAT</td>
<td>TAG</td>
<td>Transversion</td>
<td>784322</td>
<td>Aspartic acid &gt; Stop codons</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAT</td>
<td>TAG</td>
<td>Transversion</td>
<td>784324</td>
<td>Aspartic acid &gt; Stop codons</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATC</td>
<td>TTC</td>
<td>Transversion</td>
<td>784342</td>
<td>Isoleucine &gt; Phenylalanine</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ID: CP020603.1</td>
<td>100%</td>
</tr>
<tr>
<td>21</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>100%</td>
</tr>
<tr>
<td>25</td>
<td>AAC</td>
<td>TAC</td>
<td>Transversion</td>
<td>784316</td>
<td>Asparagine &gt; Tyrosine</td>
<td>Missense</td>
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</tr>
<tr>
<td></td>
<td>GAT</td>
<td>ATT</td>
<td>Transition</td>
<td>784322</td>
<td>Aspartic acid &gt; Isoleucine</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAT</td>
<td>ATT</td>
<td>Transversion</td>
<td>784323</td>
<td>Aspartic acid &gt; Isoleucine</td>
<td>Missense</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Type of mutations in the \textit{phzS} gene sequence of \textit{Pseudomonas aeruginosa} in 4 isolates

<table>
<thead>
<tr>
<th>No. Of sample</th>
<th>Wild-type</th>
<th>Mutant type</th>
<th>Type of mutation</th>
<th>Location</th>
<th>Amino acid change</th>
<th>effect</th>
<th>Sequence ID</th>
<th>Identities</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>GCC</td>
<td>ACC</td>
<td>Transition</td>
<td>8660</td>
<td>Alanine &gt; Threonine</td>
<td>Missense</td>
<td>ID: KX180139.1</td>
<td>99%</td>
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<tr>
<td>14</td>
<td></td>
<td>CGC</td>
<td>CAC</td>
<td>8697</td>
<td>Arginine &gt; Histidine</td>
<td>Missense</td>
<td>ID: CP004061.1</td>
<td>100%</td>
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<tr>
<td>15</td>
<td></td>
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<td></td>
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<td>100%</td>
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<tr>
<td>23</td>
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<td></td>
<td></td>
<td>ID: CP004061.1</td>
<td>100%</td>
</tr>
</tbody>
</table>
phenazine modifying genes. The two genes that convert phenazine-1-carboxylic acid to pyocyanin encode the putative enzymes methyltransferase and monooxygenase, respectively. Phenazine-1-carboxylic acid is first represented by the enzyme PhzM, a methyltransferase, followed by the action of the PhzS, a FAD-dependent monooxygenase enzyme. However, the overexpression of gene(s) encoding biosynthesis of pyocyanin via increasing the copy number was not found in *P. aeruginosa*.

The study aimed to detect the genetic bases of phenazine pigment production by *phzM* and *phzS* genes and confirmed by sequencing analysis.

**MATERIALS AND METHODS**

**Patients**

Out of 100 samples, 30 *P. aeruginosa* isolates were recovered from clinical and environmental sources. These samples include

<table>
<thead>
<tr>
<th>Effect of mutation</th>
<th>number</th>
<th><em>phzM</em> (%)</th>
<th><em>phzS</em> (%)</th>
<th>percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonsense</td>
<td>1</td>
<td>1 (11.11)</td>
<td>0 (0.00)</td>
<td>11.11</td>
</tr>
<tr>
<td>Missense</td>
<td>8</td>
<td>6 (66.66)</td>
<td>2 (22.22)</td>
<td>88.88</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>7</td>
<td>2</td>
<td>99.99</td>
</tr>
</tbody>
</table>

**Fig. 1.** A: Electrophoresis gel of PCR product of *phzM* genes. Lane L: DNA marker (100-1500bp); Lane (24) negative control; Lane (22) environmental isolate; Lane (5, 12, 21, 25, 26) clinical isolates give a positive result for this gene. B: Gel electrophoresis of PCR product of the *phzS* gene. Lane L: DNA marker (100-1500bp); Lane (24) negative control; Lane (3, 22) environmental isolates; Lane (2, 14, 15, 23) clinical isolates

**Fig. 2.** Phylogenetic tree based on 16S rRNA gene sequence of 4 isolates of *Pseudomonas aeruginosa* compared with sequence available in the GenBank. Nodes indicate E value
21 isolates from burn wound, 9 isolates from the hospital environment (antiseptics and soaps in burn unit). All samples and patients were obtained at Al-Hilla Surgical Teaching Hospital in Al-Hilla City/Iraq.

**Diagnosis of bacteria**

All samples that collected from different sources were cultured on MacConkey agar and incubated overnight at 37°C of plates. Diagnosis of bacteria was carried out by biochemical methods.
(oxidase, IMVIC, and nitrate reduction), as well as using phenazine production according to previous studies.\textsuperscript{7,8}

**DNA extraction**

DNA of 26 bacterial isolates collected from 21 foot ulcer and 5 environmental samples was extracted according to the Geneaid manufacturer’s instructions (Presto TM Mini Gdna bacterial Kit).

**Molecular detection of phenazine modifying genes by PCR**

Primers, PCR program, and mixtures were used to detect \textit{phzM} and \textit{phzS} genes as following: each 25 \textmu l of PCR mixture consists of forward and reverse primer (2.5 \textmu l). DNA extraction in a concentration of 0.1\textmu g/ml (5 \textmu l) and mastermix (12.5 \textmu l). The polymerase chain reaction product was detected by gel electrophoresis on 1.5 % agarose gel for 40 min. at 70V. Upstream and downstream primers for \textit{phzM} and \textit{phzS} genes were used according to \textsuperscript{[9, 10]} respectively.

**Sequencing of \textit{phzM} and \textit{phzS} genes and sequence alignment**

Four isolates of \textit{P. aeruginosa} that carry both genes \textit{phzM} and \textit{phzS} were subjected to sequencing in this study. According to the results of PCR products, the products were separated on a 2% gel electrophoresis agarose and visualized by UV light at (302 nm) after addition of Red Stain or ethidium bromide. Automatic sequencing was conducted in a biotechnology lab using DNA sequencer 3730XL, Applied Biosystems. The Basic Local Alignment Search Tool (BLAST) and BioEdit programs using the National Center Biotechnology Information (NCBI) at (http://www.ncbi.nlm.nih.gov) were used to analyze this data.

**E value and score**

The expectation value is defined to give an estimate of the number of times expected to get the same similarity coincidental and the lower
the value of E. This indicates that the degree of similarity was high between sequences which give greater confidence. The value of close to zero means that these sequences are identical and the bit score: a statistical analysis of the moral similarity and the higher value indicate that there is a high degree of similarity. If the value dropped from the class of 50 points, this similarity may not be shown.

RESULTS AND DISCUSSION

Detection of phenazine modifying genes by PCR

The two novel genes, *phzM* and *phzS* which are responsible for pyocyanin pigment
synthesis in *P. aeruginosa*. In the present study, the PCR technique was used to detect *phzM* and *phzS* genes through the use of pieces of DNA with a limited number of the oligonucleotide which acts as primer specialized virulence genes of *P. aeruginosa*. It was found that most of the clinical isolates were carried the genes responsible for pigment production with some exclusion. The results also found that two environmental isolates lacked both phenazine genes examined. Phenotypically, all the clinical isolates were found to be producing phenazine, but the environmental isolates were not the producer. Regarding *phzM*, out of 26 *P. aeruginosa* isolates, 21 (80.76%) isolates harbored

Fig. 3. Sequencing results of *phzM* genes of *Pseudomonas aeruginosa* in 4 isolates
this gene at 330 bp in PCR amplification while phzS gene represents 14 (53.84%) with 664 bp, as shown in (Fig.1). The findings of the present study were comparable with that obtained by^4, which initiates that phzM gene exists at a percentage of (84%) in *P. aeruginosa* isolates. However, similar findings were obtained by^4, which referred to the presence of the two genes is essential to create phenazine in *P. aeruginosa*. Although, the phzM gene regulating phenazine synthesis was found in (80.76%) of present tested isolates, that may be due to the diversity of these isolates^11. According to mutant strain analysis of these two genes, it was postulated that mutant strain demonstrates variable phenazine production^4.

### Sequencing of phzM and phzS genes

In the present study genotypic variation of environmental and clinical isolates of this bacterium was studied by sequencing of phzM and phzS genes. The relationships of genetic evolution of pseudomonas isolates were examined by comparing the analysis of the sequence with the NCBI. On the basis of the evidence by sequencing, it has been postulated that all the four isolates from clinical and environmental samples belong to the species *Pseudomonas aeruginosa* (Fig.2). The analysis of phylogenetic sequences has not revealed considerable diversity in environmental and clinical isolates (Fig.2). The findings of phzM gene sequence demonstrated that there were 7
mutations with 99% identities in 4 isolates as shown in (table 1) and (Fig.3). While for the phzS gene 2 mutations with identities 99% were detected by sequencing in the same isolates (table 2) and (Fig.4). The current results also appeared that P. aeruginosa isolates collected from environmental and clinical sources have a core genome which was highly conserved. However, these isolates tended to be variable in regards to the presence of regions involved in the phenazine phenotype.

Effect of mutations in phzM and phzS genes on phenazine production

To assess the impact of the biosynthesis modifying genes phzM and phzS on phenazine production in pseudomonas isolates with mutations in these genes. The isolates showed uncommon pigment phenotypes when they were cultured. While blue cultures of wild-type were attributable to the pyocyanin production, this finding was in agreement with the results conducted by. It was interesting to mention that the existence of dual operons regulating biosynthesis of phenazine makes this bacterium more flexibility in modulating the number of phenazine compounds. It has been suggested that the variation in phenazine production might be ascribed to growth phase or in response to signals from the environment. Mutations affect the phzM and phzS genes through the creation of change in the gene sequence. The results in the table (1) showed that 1(11.11%) nonsense mutation
3. Isolate NO. 15
Pseudomonas aeruginosa VRFPA04, complete genome
Sequence ID: CP008789, Length: 6818030, Number of Matches: 1
Related Information
Range 1: 5925621 to 5926185

GenBank Graphics

Score | Expect | Identities | Gaps | Strand
--- | --- | --- | --- | ---
1020 bits (1130) | 0.0 | 565/565 (100%) | 0/565 (0%) | Plus/Plus

Query 361
GGCTATCCGATCTCAGCGCAACGGCCGAGGCAAAGTACGTCGTGGACTGCGGTTG 420

Subject 5925681
GGCTATCCGATCTCAGCGCAACGGCCGAGGCAAAGTACGTCGTGGACTGCGGTTG 5926040

CDS: hypothetical pro 218
AYPI SAR HAAEGKSLVNW V C

CDS: Putative 1 141
MVP SAAV GLQLDNEADWRN G

Query 421
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Subject 5926041
CATGGTCGAGCCGCGGTGAGCTGACAACTGGCACACCATGCGAACGG 5926100

CDS: hypothetical pro 238
MVP SAAV GLQLDNEADWRN G

CDS: Putative 1 161
RLEDVLPFFFAADVWDLGRFD I R

Query 481
ACGCCGTGGAAGCTTTGCTCGCGGATCGTGGCATGGGCTGTTGCTGACATCCG 540

Subject 5926101
ACGCCGTGGAAGCTTTGCTCGCGGATCGTGGCATGGGCTGTTGCTGACATCCG 5926160

CDS: hypothetical pro 258
RLEDVLPFFFAADVWDLGRFD I R

CDS: Putative 1 181
DLLTRNQL

Query 541
CGACCTGTGACCGCAACCAGTTG 565

Subject 5926161
CGACCTGTGACCGCAACCAGTTG 5926185

CDS: hypothetical pro 278
DLLTRNQL

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which leads to change the code of amino acid stop codon, causing dysfunction of the protein. Furthermore, there is 8(88.88%) missense mutation in both genes, this type of mutation influences the phenotype because they lead to substitution of amino acids and thus in protein. An amino acid can be replaced with another amino acid that has similar chemical characteristics, therefore the protein could normally work. There is also an amino acid encoded by more than one code which could result in mutation. Nevertheless, this mutation does not produce any change in the translation.

**CONCLUSION**

From the present study, it seems that *P. aeruginosa* isolates of clinical and environmental sources have a very preserved genome core. The
sequence of bacterial genome gives significant evidence to appreciate the regulatory and metabolic network that link chromosomal genes. Although the pseudomonas isolates have mutations in phzM and phzS genes, it has been proposed that these mutations have no role in the pathogenesis. In this site, the crucial part of \( P.\ aeruginosa \) virulence looks to be the regulation of gene expression instead of the existence or nonexistence of genes.

**ACKNOWLEDGEMENT**

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**REFERENCES**