JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2018. Vol. **12**(1), p. 29-40

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

Huda Hadi Al-Hasnawy

The University of Babylon, College of Medicine, Department of Microbiology, Iraq.

http://dx.doi.org/10.22207/JPAM.12.1.05

(Received: 08 January 2018; accepted: 20 February 2018)

Study the sequencing of *phz*M and *phz*S 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 *phz*M and *phz*S gene were present in 21(80.76%), 14(53.84%) respectively. Sequencing of *phz*M and *phz*S 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[1]. *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[2]. It is a marine and soil organism and an opportunistic human pathogen able to infect different sites and tissues[3]. 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[4]. 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[5]. The biosynthetic genes of phenazine are organized in one operon, *phz ABCDEFG* in all Pseudomonas species with the exception of *P.aeruginosa* 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

* To whom all correspondence should be addressed.
E-mail:hudashmm@gmail.com

Table 1. Type of mutations in the *phz*M gene sequence of *Pseudomonas aeruginosa* in 4 isolates

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

Table 2. Type of mutations in the phzS gene sequence of *Pseudomonas aeruginosa* in 4 isolates

No. Of sample	Wild-type	Mutant type	Type of mutation	Location	Amino acid change	effect	Sequence ID	Identities
2	GCC	ACC	Transition	8660	Alanine> Threonine	Missense	ID: KX180139.1	99%
	CGC	CAC	Transition	8697	Arginine> Histidine	Missense		
14							ID: CP004061.1	100%
15							ID: CP008739.2	100%
23							ID: CP004061.1	100%

phenazine modifying genes**.** The two genes that convert phenazine-1-carboxylic acid to pyocyanin encode the putative enzymes methyltransferase and monooxygenase, respectively[6]. 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*[4]. The study aimed to detect the genetic bases of phenazine pigment production by *phz*M and *phz*S 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 3. Effect of the type and percentage of mutation on *phz*M, phzS genes

Effect of mutation	number	*phz*M (%)	*phz*S (%)	percentage
nonsense	1	1(11.11)	0 (0.00)	11.11
Missense	8	6 (66.66)	2 (22.22)	88.88
Total	9	7	2	99.99

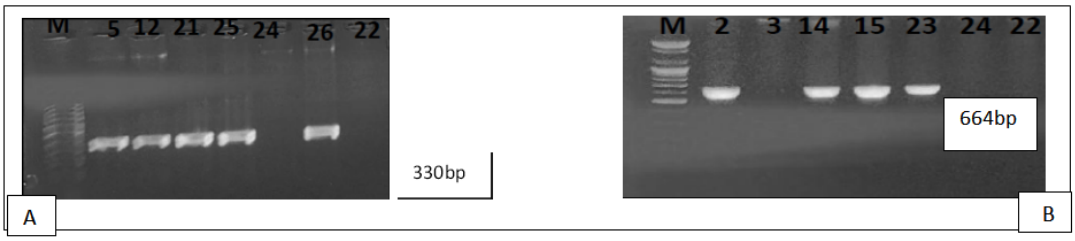

Fig. 1. A: Electrophoresis gel of PCR product of *phz*M genes. Lane L: DNA marker (100-1500bp); Lane (24) negative control; Lane (22) environmental isolate; Lane (5, 12, 21, 25, 26) clinical isolates give a positive result for this gene. B: Gel electrophoresis of PCR product of the *phz*S gene. Lane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

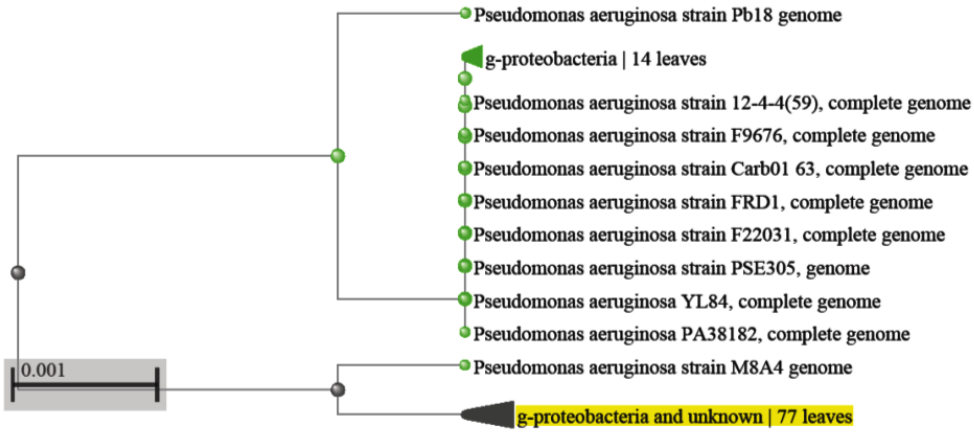

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence of 4 isolates of Pseudomonas aeruginosa compared with sequence available in the GenBank. Nodes indicate E value

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

1- Isolate Number 5

Pseudomonas aeruginosa strain E6130952, complete genome

Sequence ID: CP020603.1 Length: 7040952 Number of Matches: 1
Related Information
Range 1: 784047 to 784332 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
499 bits(552)	2e-137	282/286(99%)	0/286(0%)	Plus/Plus

```
CDS: Putative 1       40      K  A  I  L  Q  A  E  P  S  A  R  G  V  M  L  D  R  E  G
S

Query                 121
AGGCCATCCTGCAGGCCGAGCCCAGCGCCCGGGGCGTGATGCTCGACCGCGAGGGTTCCC  180

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct                 784167
AGGCCATCCTGCAGGCCGAGCCCAGCGCCCGGGGCGTGATGCTCGACCGCGAGGGTTCCC  784226

CDS:putative phenazi  183     K  A  I  L  Q  A  E  P  S  A  R  G  V  M  L  D  R  E  G
S


CDS: Putative 1       60      L  G  V  A  R  D  N  L  S  S  L  L  A  G  E  R  V  S  L
V

Query                 181
TCGGCGTGGCCCGCGACAACCTCTCCAGCCTGTTGGCAGGGGAGCGCGTCAGCCTGGTGG  240

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct                 784227
TCGGCGTGGCCCGCGACAACCTCTCCAGCCTGTTGGCAGGGGAGCGCGTCAGCCTGGTGG  784286

CDS:putative phenazi  203     L  G  V  A  R  D  N  L  S  S  L  L  A  G  E  R  V  S  L
V


CDS: Putative 1       80      G  G  D  M  L  Q  E  V  P  S  N  G  *  F  Y  L

Query                 241     GCGGCGACATGCTGCAAGAGGTGCCGTCCAACGGATAGTTCTACCT  286

                              ||||||||||||||||||||||||||||||||  | |||||||

Sbjct                 784287  GCGGCGACATGCTGCAAGAGGTGCCGTCCAACGGCGATATCTACCT  784332

CDS:putative phenazi  223     G  G  D  M  L  Q  E  V  P  S  N  G  D  I  Y  L
```

(oxidase, IMVIC, and nitrate reduction), as well as using phenazine production according to previous studies[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 *phz*M and *phz*S genes as following; each 25 µl of PCR mixture consists of forward and reverse primer (2.5 µl). DNA extraction in a concentration of 0.1µg/ml (5 µl) and mastermix (12.5 µl). 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 *phz*M and *phz*S genes were used according to [9, 10] respectively.

Sequencing of *phz*m and *phz*s genes and sequence alignment

Four isolates of *P. aeruginosa* that carry both genes *phz* M and *phz*S 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

3-Isolate No. 21

Pseudomonas aeruginosa strain E6130952, complete genome
Sequence ID: CP020603.1Length: 7040952Number of Matches: 1
Related Information
Range 1: 784050 to 784320GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
489 bits(542)	1e-134	271/271(100%)	0/271(0%)	Plus/Plus

```
CDS: Putative 1       40      A  I  L  Q  A  E  P  S  A  R  G  V  M  L  D  R  E  G  S
L

Query                 121
CCATCCTGCAGGCCGAGCCCAGCGCCCGGGGCGTGATGCTCGACCGCGAGGGTTCCCTCG  180

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct                 784170
CCATCCTGCAGGCCGAGCCCAGCGCCCGGGGCGTGATGCTCGACCGCGAGGGTTCCCTCG  784229

CDS:putative phenazi  184     A  I  L  Q  A  E  P  S  A  R  G  V  M  L  D  R  E  G  S
L


CDS: Putative 1       60      G  V  A  R  D  N  L  S  S  L  L  A  G  E  R  V  S  L  V
G

Query                 181
GCGTGGCCCGCGACAACCTCTCCAGCCTGTTGGCAGGGGAGCGCGTCAGCCTGGTGGGCG  240

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct                 784230
GCGTGGCCCGCGACAACCTCTCCAGCCTGTTGGCAGGGGAGCGCGTCAGCCTGGTGGGCG  784289

CDS:putative phenazi  204     G  V  A  R  D  N  L  S  S  L  L  A  G  E  R  V  S  L  V
G


CDS: Putative 1       80      G  D  M  L  Q  E  V  P  S  N  G

Query                 241     GCGACATGCTGCAAGAGGTGCCGTCCAACGG  271
                              ||||||||||||||||||||||||||||||||
Sbjct                 784290  GCGACATGCTGCAAGAGGTGCCGTCCAACGG  784320

CDS:putative phenazi  224     G  D  M  L  Q  E  V  P  S  N  G
```

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 be the producer. Regarding *phz*M, out of 26 *P. aeuroginosa* isolates, 21 (80.76%) isolates harbored

Fig. 3. Sequencing results of *phz*M genes of *Pseudomonas aeruginosa* in 4 isolates

this gene at 330 bp in PCR amplification while *phz*S 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[9], which initiates that *phz*M 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 *phz*M 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 *phz*m and *phz*s genes

In the present study genotypic variation of environmental and clinical isolates of this bacterium was studied by sequencing of *phz*M and *phz*S 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 *phz*M gene sequence demonstrated that there were 7


1-Isolate NO.2

Pseudomonas aeruginosa strain PA1201 phenazine biosynthesis putative Flavin-containing monooxygenase (phzS) genes, complete cds

Sequence ID: KX180139.1 Length: 9526 Number of Matches: 1

Related Information

Range 1: 8608 to 9175 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1016 bits(1126)	0.0	566/568(99%)	0/568(0%)	Plus/Plus

```
CDS: Putative 1       1       A  Y  P  Q  Y  S  I  H  R  G  E  L  Q  M  I  L  L  T  A
V

Query                 1
CGCCTATCCGCAGTACTCGATCCATCGCGGCGAACTGCAGATGATCCTGCTCACCGCGGT  60

                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
|||||||

Sbjct                 8608
CGCCTATCCGCAGTACTCGATCCATCGCGGCGAACTGCAGATGATCCTGCTCGCCGCGGT  8667

CDS:putative flavin-  98      A  Y  P  Q  Y  S  I  H  R  G  E  L  Q  M  I  L  L  A  A
V


CDS: Putative 1       21      R  E  R  L  G  Q  Q  A  V  H  T  G  L  G  V  E  R  I  E
E

Query                 61
GCGCGAGCGTCTCGGCCAACAGGCGGTACACACCGGTCTCGGCGTGGAGCGCATCGAAGA  120

                          ||||||||||||||||||||||||||||||
||||||||||||||||||||||||||||||

Sbjct                 8668
GCGCGAGCGTCTCGGCCAACAGGCGGTACGCACCGGTCTCGGCGTGGAGCGCATCGAAGA  8727

CDS:putative flavin-  118     R  E  R  L  G  Q  Q  A  V  R  T  G  L  G  V  E  R  I  E
E
```

mutations with 99% identities in 4 isolates as shown in (table 1) and (Fig.3), While for the *phz*S 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 *phz*M and *phz*S genes on phenazine production

To assess the impact of the biosynthesis modifying genes *phz*M and *phz*S 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[4]. 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 *phz*M and *phz*S genes through the creation of change in the gene sequence. The results in the table (1) showed that 1(11.11%) nonsense mutation


2-Isolate NO. 14
Pseudomonas aeruginosa B136-33, complete genome
Sequence ID: CP004061.1Length: 6421010Number of Matches: 1
Related Information
Range 1: 766828 to 767367GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
975 bits(1080)	0.0	540/540(100%)	0/540(0%)	Plus/Minus

Hypothetical protein

CDS: Putative 1 1 G E L Q M I L L T A V R E R L G Q Q A

Query 1
GCGGCGAACTGCAGATGATCCTGCTCACGCGGTGCGCGAGCGTCTGGCCAACAGGCGG 60

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 767367
GCGGCGAACTGCAGATGATCCTGCTCACGCGGTGCGCGAGCGTCTGGCCAACAGGCGG 767308

CDS:hypothetical pro 106 R G E L Q M I L L T A V R E R L G Q Q A

CDS: Putative 1 20 V H T G L G V E R I E E R D G R V L I G

Query 61
TACACACCGGTCTCGGCGTGGAGCGCATCGAAGAGCGCGACGGCCGCGTACTGATCGGCG 120

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 767307
TACACACCGGTCTCGGCGTGGAGCGCATCGAAGAGCGCGACGGCCGCGTACTGATCGGCG 767248

CDS:hypothetical pro 126 V H T G L G V E R I E E R D G R V L I G

3-Isolate NO.15
Pseudomonas aeruginosa VRFPA04, complete genome
Sequence ID: CP008739.2 Length: 6818030 Number of Matches: 1
Related Information
Range 1: 5925621 to 5926185 GenBank Graphics Next Match Previous Match

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

```
Query                  361
CGCCTATCCGATCTCGGCGCGCCACGCGGCCGAAGGCAAGTCGCTGGTGAACTGGGTGTG  420

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct                  5925981
CGCCTATCCGATCTCGGCGCGCCACGCGGCCGAAGGCAAGTCGCTGGTGAACTGGGTGTG  5926040

CDS:hypothetical pro   218        A  Y  P  I  S  A  R  H  A  A  E  G  K  S  L  V  N  W
V  C


CDS: Putative 1        141        M  V  P  S  A  A  V  G  Q  L  D  N  E  A  D  W  N  R
N  G

Query                  421
CATGGTGCCGAGCGCCGCCGTCGGCCAGCTCGACAACGAGGCCGACTGGAACCGCAACGG  480

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct                  5926041
CATGGTGCCGAGCGCCGCCGTCGGCCAGCTCGACAACGAGGCCGACTGGAACCGCAACGG  5926100

CDS:hypothetical pro   238        M  V  P  S  A  A  V  G  Q  L  D  N  E  A  D  W  N  R
N  G


CDS: Putative 1        161        R  L  E  D  V  L  P  F  F  A  D  W  D  L  G  W  F  D
I  R

Query                  481
ACGCCTGGAAGACGTGTTGCCGTTCTTCGCCGACTGGGACCTGGGCTGGTTCGACATCCG  540

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct                  5926101
ACGCCTGGAAGACGTGTTGCCGTTCTTCGCCGACTGGGACCTGGGCTGGTTCGACATCCG  5926160

CDS:hypothetical pro   258        R  L  E  D  V  L  P  F  F  A  D  W  D  L  G  W  F  D
I  R


CDS: Putative 1        181        D  L  L  T  R  N  Q  L

Query                  541      CGACCTGCTGACCCGCAACCAGTTG  565
                                |||||||||||||||||||||||||
Sbjct                  5926161  CGACCTGCTGACCCGCAACCAGTTG  5926185

CDS:hypothetical pro   278        D  L  L  T  R  N  Q  L
```

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

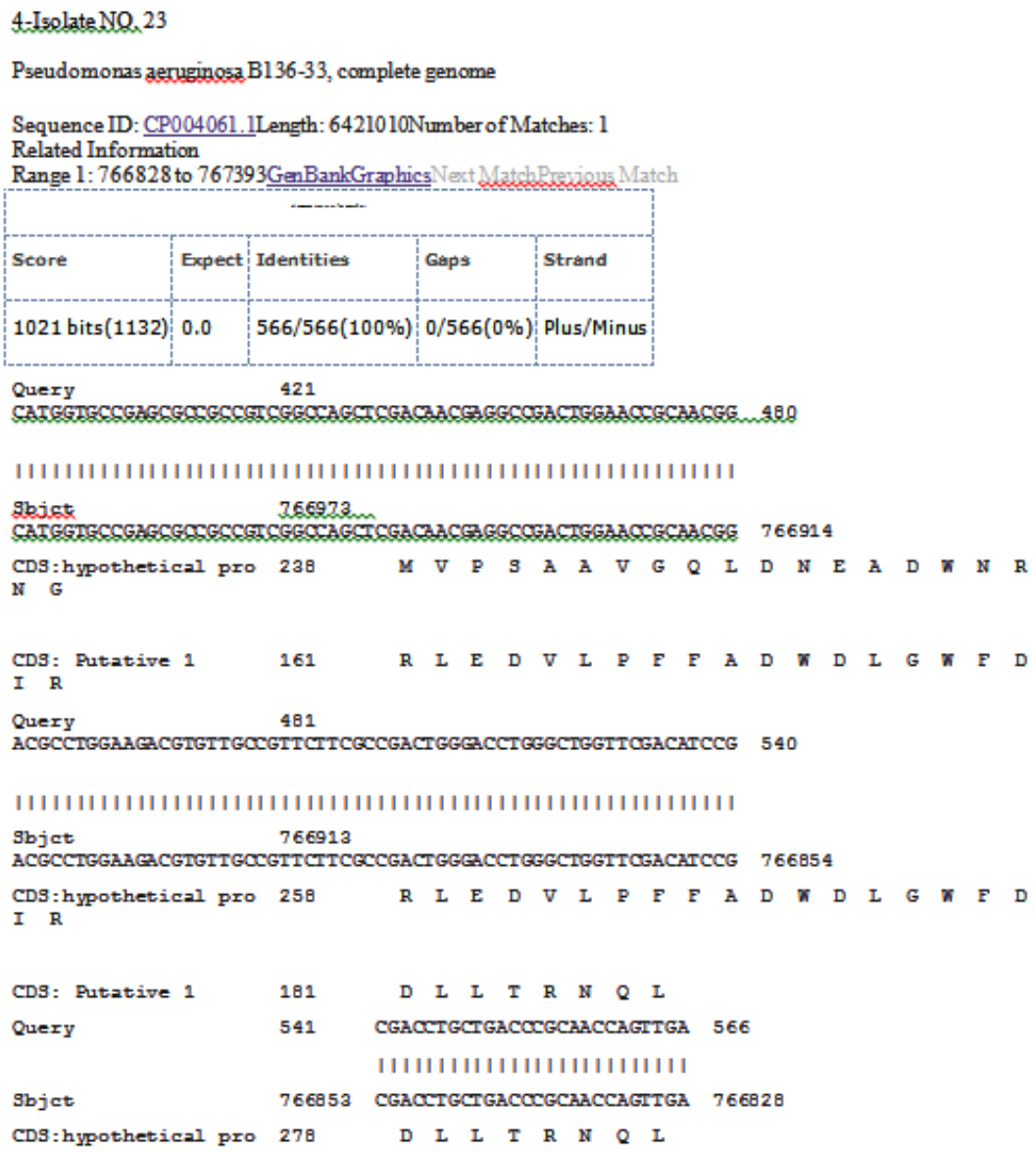

Fig. 4. Sequencing results of *phz*S genes of *Pseudomonas aeruginosa* in 4 isolates

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 *phz*M and *phz*S 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

I am thankful the Microbiology Department, Medicine College of Babylon University / Iraq for the facilities provided in the completion of the work. I am also thankful Hiba Jasim for her cooperation.

REFERENCES

1. Gross, H. and Loper, J.E. Genomics of secondary metabolite production by *Pseudomonas* spp. *Natural Products Reports,* 2009; **26**: 1408-46.
2. Shirley, F., John, P., Morrissey, Fergal O'Gara and Fidelma Boyd E. Genome Diversity of Pseudomonas aeruginosa isolates from Cystic Fibrosis Patients and the Hospital Environment. *Journal of clinical microbiology*, 2004; **42**(12): 5783–5792.
3. Lyczak, J.B., Cannon, C.L. and Pier, G.B. Establishment of *Pseudomonasaeruginosa* infection.: lessons from a versatile opportunist. *Microbes & Infection,* 2000; **2**: 1051-1060.
4. Mavrodi, D.V., Bonsall, R.F., Deleney, S.M., Soule, M.J., Phillips, G. and Thomashow, L.S. Functional Analysis of Genes for Biosynthesis of Pyocyanin and Phenazine-1-Carboxamide from *Pseudomonas aeruginosa* PAO1. *Journal of Bacteriology*, 2001; **183**(21): 6454-6465
5. Preetha, R., Jose, S., Prathapan, S., Vijayan, K.K., Jayaprakash, N.S., Philipm, R. and Bright Singh, I.S. An inhibitory compound produced by *Pseudomonas* with effectiveness on *Vibrio harveyi*. *Aquaculture Research,* 2010; **41**: 1452-1461.
6. Mavrodi, D.V., Ksenzenko, V.N., Bonsall, R.F., Cook, R.J., Boronin, A.M., Thomashow, L.S. A seven-gene locus for the synthesis of phenazine–1- carboxylic acid by Pseudomonas fluorescens. *Journal of Bacteriology*, 1998; **180**: 2541–2548.
7. Forbes, B.A. Daniel, F.S. and Alice, S.W: Bailey and Scott's diagnostic microbiology, 12th. edn. USA: Mosby Elsevier Company, 2007.
8. Frank, L. H., and DeMoss, R. D. On the biosynthesis of pyocyanine. *J Bacteriol*, 1959; **77:** 776–782.
9. Shi, H., Trinh, Q., Xu, W., Zhai, B., Luo, Y. and Huang, K. A universal primer multiplex PCR method for typing of toxinogenic Pseudomonas aeruginosa. *Appl Microbiol Biotechnol*, 2012; **95**: 1579– 1587.
10. Nowroozi, J., Sepahi, A.A. and Rashnonejad, A. Pyocyanine biosynthetic genes in clinical and environmental isolates of *Pseudomonas aeruginosa* and detection of pyocyanine's antimicrobial effects with or without colloidal silver nanoparticles. *Cell Journal* (Yakhteh), 2011; **14**(1): 7-18.
11. Chieda, Y., Iiyama, K., Lee, J.M., Kusakabe, T., Yasunaga-Aoki, C. and Shimizu, S. Inactivation of pyocyanin synthesis genes have no effect on the virulence of *Pseudomonas aeruginosa* PAO1 toward the silkworm, *Bombyx mori*. *FEMS Microbiology Letters,* 2008; **278**: 101-107.