Original Article

Isolation Of Pseudomonas Fluorescens From Erbil Soils And Screening For The Presence Of Siderophores Biosynthesis Genes

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DOI: 10.47750/pnr.2023.14.04.17

Abstract

The current study was aimed to isolate and identify Fluorescent Pseudomonads from Erbil soil and then screening for Siderophores biosynthesis genes. Soil samples were collected from different location in Erbil governorate. A total of 18 isolates of Pseudomonas fluorescens were isolated on enriched and selective King B medium and they identified using API20E, Vitek2 morphological features, cultural, biochemical characteristics, and 16S rRNA gene sequencing technique. Plant growth promoting traits of the isolates were also studied such as IAA, siderophore and HCN production. All isolates exhibited high potential of IAA, HCN and siderophore production. We selected different isolate for screen the presence of Iron solubilizing gene using specific primers with PCR technique. All isolates resolute for their iron solubilizing efficiency and all of them showed iron solubilizing activity. Isolate (Cpf3) were the most efficient isolate, while (Dpf16) were the least efficient isolates in Fe-solubilization. psiB and psiG genes were selected to screen for the presence of iron solubilizing using specific primers with PCR technique. The results showed positive reactions and all isolates contained iron solubilizing genes.

Keywords: Pseudomonas fluorescens, iron solubilizing efficiency, iron solubilizing genes.

INTRODUCTION

Despite of the amount of total iron is high 5% in earth crust but the availability is very low. Wintz et al., 1978. It is the fourth common element in the Earth's crust is iron, is not easily accessible to most forms of life. Iron is essential to virtually all life forms, and only a few bacteria can substitute it with other metals. Rhizospheres are the main source of Fe for plants; Plant roots do not have direct access to Fe, despite its abundance in the earth's crust according to Marschner, 1995. The photosynthesis and chlorophyll synthesis of plants also require iron. The distribution of plant species and the amount of iron in the soil, which affects crop yields and nutrient quality, are characteristics of natural ecosystems. When there is insufficient iron intake, growth is slowed down and fitness is decreased.Wolfgang, 2020. Wintz et al., 2002 pointed out that the plant absorbs the iron ion mainly on a divalent iron image and can be absorbed by the plant in an organic compound such as (chelated iron). Biological organisms boost their chances of surviving in low-iron environments by secreting siderophores, which are ligands that bind to iron. According to O'Sullivan and O'Gara (1992), iron firmly controls the synthesis of these "low-molecular-mass, fundamentally ferric specific" ligands. Therefore, siderophores may directly induce the development of new anti-microbial compounds when these elements become more available to bacteria (Duffy and Defago, 1999). Despite its importance, iron is in short supply in the rhizosphere due to competition between plant roots, bacteria, and fungus) Guerinot and Ying, 1994). Pseudomonas is one of the most prevalent bacterial genera in the plant rhizosphere (Philippot et al., 2013). Pseudomonas fluorescens bacteria present in various plants rhizosphere and act as PGPR that lead to increasing nutrients (Klopper et al ., 1988) Some Pseudomonas strains support plant growth by improving nutrient and iron uptake, and others defend plants against pathogen infection through mechanisms like competition, antibiosis, and the induction of systemic resistance (ISR) (Chowdhury et al., 2015). Some studies show that Pseudomonas bacteria present in the root surroundings of plants are among the best types of bacteria that stimulate plant growth. Lata et al., 2002. Pseudomonas fluorescens is one of the most beneficial rhizosphere bacteria mostly used to control broad range of soil borne plant pathogens. Al-Waily et al., 2018. It is

ubiquitous, present not only in soil but even on plant surfaces with iron deficiency, P.fluorescens produces a strong mixture of low molecular weight siderophores like pyoverdine, which boosts the organism's ecological competency (Mirleau et al, 2001). The current study aimed to isolate and characterize P.fluorescens from Erbil governorate soil and to evaluate the ability of P.fluorescens isolates (psiB and psiG genes) in iron solubilization and then screening for the presence of iron solubilizing genes.

MATERIAIS AND METHODS

Collections of Soil Samples

Sampling was conducted from different locations in Erbil governorate-Iraq involved: (Grdarasha, Khabat, Jamadubze, Mala-Omer, Zargazawy, Bawakhalan, Choman, Harer, Makhmoor, Qushtapa, Dibaga, Spilk and Warte on October 2020. The soils were transported to the Microbiology Laboratory at the college of Agriculture Engineering Science at Salahaddin University-Erbil. Samples were collected in sterilized bags and kept at 4°C and processed a few days after sampling.

Isolations and Identifications of Bacterial strains

Isolation of iron-solubilizing bacteria was achieved by serial dilutions of soil samples, and the bacteria were subsequently cultivated onto Biotite plates with King's B medium agar. The following ingredients were used: proteose peptone (20.0), Biotite (2), dipotassium hydrogen phosphate (1.50), magnesium sulfate heptahydrate (1.50), agar (20.0), and a final pH of 7.20, all at 28C for 72 hours. During incubation at 28°C, tests were conducted using microscopy, morphology, biochemistry, and physiology in accordance with Bergey's Manual for Determinative Bacteriology. included (cell shape, gram stain, Colony morphology, color, size, spore formation, arrangement, motility, colony characters, flagellum observation, aerobic test, pigmentation, oxidase and catalase test, Gelatin liquefaction, starch hydrolysis, glucose fermentation, arginine dihydrolase, levan production, nitrate reduction, utilization of trehalose and tryptophan, growth at 4˚C and 41˚C, and different sugar utilization (Loekas et al., 2011) and (Gulez et al., 2014). All isolates were also identified using API 20e in accordance with API 20e cod book, and using the API 20e online cod book application system, the findings were analyzed. Additionally, according to the Biomerieux-diagnostics procedure, the VITEK 2 technology was used to identify the examined bacteria based to the species level.

Based on the 16S rDNA partial sequence, each strain was molecularly identified. The following universal bacterial primers were used for DNA extraction and amplification: 8F (5'-AGAGTTTGATCCTGGCTCAG-3')and 1492R(5'-ACGGCTACCTTGTTACGACTT-3').The PCR was conducted using Lucigen EconoTaq Plus Green 2X master mix (Lucigen Corp.) at the following cycling parameters: Initiate denaturation at 95°C for five minutes, followed by 31 cycles of 94°C for one minute, 57°C for 45 seconds, and 70°C for two minutes, followed by a final extension at 70°C for ten minutes. To identify the bacterial taxa of each isolate, all sequences were compared against the ribosomal database project type strains. Nucleotide basic local alignment and search tool (BLAST) was used to align the obtained sequences with reference RNA sequences from a database at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov).

Molecular identifications of siderophore biosynthesis genes.

The genomics of bacteria were extracted using PureLink Genomic DNA Kit protocols for gram negative bacterial samples. The NanoDrop method was applied in order to confirm the quantity and quality of the isolated genomes. We amplified the bacterial genomic data using PCR and one of the universal primers for 16S rRNA genes (Forward: 5' AGAGTTTGATCMTGGCTCAG3',and Revers:5'CTGCTGCSYCCCGTAG3'). (Waldeisen JR et al., 2011). To confirm the size of the amplified gene, PCR products were visualized on a 1.5 percent agarose gel stained with ethidium bromide under UV light. Prior to bidirectional sequencing with primers 27F and 1392R, Purification of PCR products was carried out using EXOSAP-IT (Ambion, CA) (Srinivasan et al., 2015). The Oligomer Biotechnology sequencing service (https://oligomer.com.tr/) was used to generate the Sanger sequences. ChromasPro was used to analyze the generated sequences.

The primers and sequences of all desired genes from P. flourences were designed by https://blast.ncbi.nlm.nih.gov/Blast.cgi. (Fig). The genes of bacteria were amplified using the PCR technique. The PCR protocol in 20uL reactions was set up in accordance with the primer milting temperatures (Tm). The temperatures needed

for primer annealing to occur in the bacterial psiB and psiG genomes were 53 and 54.5 degrees Celsius, respectively. A 1.5 percent agarose gel and UV detection with ethidium bromide were used to confirm the sizes of the amplified genes.

TCCTCACCAGCTCTCATTTCGACGACGTGCTGCACGAGTCGTTGATGTACAATGGCATGCACAAGGTCGA
GGAAATCGAGCGCCGCTCCGGCTTCCAGTCCAAGGCGCACTTGCGCATGCTGCTCAGCGAAGTGCGCGCG
GCGGTGACCGATTTCGAACCGATGAAGGCCATGGGCAATACACTTCACCTTCCTCGGCTACGAAGAGTTC
GTGGTCACCGATCAGGCCGAGGGCATACGTGCCGAAGAAAAGAACGAAATCAAAGGCTTCCTGGAATGG
CTGGTGGGCACATCAGGCAGACACTTCACCTTCCTCGGCTACGAAGAGTTCGTGGTCACCGATCAGGCCG
AGTCATTCCGAGGCGGGCACATCGAGTATGACCAGAGCTCGTTCCTTGGCCTGACCAAGAAATCAGGGCG
CCCTGCGCACCGGCATGACCTACGATGACACGCGCATCGAAAATTATGCCGACCCTACCGATGAACTACC
TGCGCGAACCGAAACTGCTGACCTTCGCCAAGGCTGCGCACTTATGGAACCCCAAGCCGCGTGCACCGTC
CCGCTTACCCTGACTACGTGTCGATCCGTGCATCAGGCCGAATCGACGCTGATGGCAAAGTCATCAAGGA
ATGCCGTTTCATGGGCCTGTTATCAACACGATGACACCTTATCGGTGTACGGCGAAACCTTCGGACCATCC
CGTACATTCGTTCAAGCAGGAACAAGGTCTTAGAAATCGAGCGCCGCTCCGGCTTCCAGTCCAAGGCGCA
CGTCAGACCGTCCTGGGCAAGGAACTGGCGCAGGTCCTGGAAGTACTGCCACGGGACGACCAATCATTCC
GACGGACTGCGAGTTCTGGACCTTCTTCTCCGAGTCCGTGCTGGCCCGCGTTATCAGGCTGACAGATCCAG
GAACGCACCACGATTCAAATGTTCGTCA

937 bp

1-psiB gene

Fw (AGGAGTGGTCGAGAGTAAAGCT)

Re (TGACGAACATTTGAATCGTGGT)

ATCGACGCTGATGGCAAAGTCATCAAGGAATGCCGTTTCATGGGCCTGTTATCAACACGATCACCCTACC
TTCCTCGGCTACGAAGAGTTCGTGGTCACCGATCAGGCCGAGGGCATACGTGCCGAAGATGAGAACGAA
ATCAAAGGCAACGTGGAATGGCTGGTGGGCACATCAGGCAGACACTTCACCTTCCTCGGCTACGAAGAGT
TCGTGGTCACCGATCAGGCCGAGTCATTCCGAGACACCTTATCGGTGTACGGCGAAACCTTCGGACCATC
CCGTACATTCGTTCAAGCAGGAAACGGTCACATCGAGTATGACCAGAGCTCGTTCCTTGGCCTGACCAAG
AAATCAGGGCGCCCTATACACCGGCATGACCTACGATGACACGCGCATCGAAAATTATGCCGACCCTACC
GAGCTGCGCTGACCGTTGAAACCTTCGGCGAAGCCCAGGGCACCAACGTACTGGAGCGCACTGGCGGATT
TCCCTAAAGGCTTCCCCGCCGGTTATCGCGAGCGTTTCGCAGAATGCTAGCAGCACTCCAATTATTGTCCA
CGGTACATCAGG

571 bp

2-psiG gene

FW (TAGCTGCGACTACCGTTTCA)

RE (CCTGATGTACCGTGGACAAT)

Plant Growth Promoting Activities

Analyzing Indole Acetic Acid (IAA)

The Salkowski method was used to examine the formation of IAA. The development of a red color was a sign that IAA was present in the culture media.

Hydrogen Cyanide (HCN) detection

Lorck and Castric were used to determine HCN formation. Using King's B agar plates as a platform for producing HCN, isolates were streaked into solution.

Siderophore Production

Chrome Azurole S (CAS) agar was used to test and evaluate siderophore production.

Iron solubilizing efficiency test

The appearance of clearing zones on basal medium supplemented with an insoluble iron source was an indication of iron solubilization. The basic medium (NaCl, 0.1 g, $MgSO_4•7H_2O$, 0.5 g, $CaCl_2•2H_2O$, 0.1 g, KH_2PO_4 (3 mM P), Fe_2O_4, 0.5 mg, $MnSO_4•H_2O$, 1.56 mg, $ZnSO_4•7H_2O$, 1.40 mg, vitamin B12, 2 g, and N sources) was added to dishes with the bacterial culture discs to remove any soluble iron, inorganic iron sources were washed three times in accordance with the advice of Reyes et al. (1999) and Gadagi and Sa (2002). As a pH indicator, bromocresol green (BCG) was applied as follows: 1 L of Reyes medium was mixed with 5 mL of a BCG stock solution (0.5% BCG in 70% ethanol, which was then pH-adjusted to 6.5 with 1 N KOH). 15 days were spent incubating the dishes at 30°C in a bacteriological incubator. Through the fifteenth day of incubation, $Fe(OH)_2$ solubilization in solid medium was evaluated. Following a 7-day measurement of the solubilization zones (also known as the clear zone) surrounding the bacterial colonies, the solubilization index was calculated using the formula shown below (Berraqueiro et al., 1976):

RESUITS AND DISCUSSION

Isolation and Identification of bacteria

To isolate bacteria from various locations in the city of Erbil, samples were collected. Only 18 isolates were selected from the 104 soil samples on the basis of their similarity in morphological and biochemical assays on the biotite-containing King's B medium. Microscopical and biochemical analysis of the isolates revealed that they were coccobacilli, motile, nonspore former, aerobic, gram negative, produced catalase, gelatinase, arginine dihydrolase, oxidase, and fluorescent pigments on King B medium under UV. Starch and urea hydrolysis, and that they were able to use trehalose, produce levan from sucrose, reduce nitrate levels, and grow at 4 and 41 degrees Celsius (Table 1 and 2). Several carbon sources were used by different strains, including xylose, lactose, fructose, melibiose, glycerol, Larabinose, ribose, D-arabinose, xylitol, malonate, sorbose, sorbitol, trehalose, mannitol, and glucosamine. However, maltose and sucrose were not used by these strains. Based on the results, P. fluorescense provided extensive phenotypic characterization (Patel et al., 2013).

The API20E approach was used to identify bacteria at the genus stage, as seen in (Figure 1). The results showed that the bacteria from culture media belonged to the genus Pseudomonas spp. (Woolfrey et al., 1981). The Vitek2 compact technique was used to identify bacteria at the species level. The results showed that the bacteria was P. fluorescens (Figure 2). The majority of microorganisms and their antibiotic susceptibility may be automatically identified using the Vitek2 compact. (https://www.biomerieux-diagnostics.com/).

Fig 1:- Results of API 20E technique for identifications bacteria.

It was necessary to confirm both the species and strain of bacteria by using a molecular identification technique. The amplified genes were visualized on agarose gels using a 16s rRNA universal primer. The amplified genes were around 620 bp in length, which is the same size as Pseudomonas fluorescens' 16S rRNA gene. (Lane 3 in Figure 3) (Waldeisen JR et al., 2011; Patel et al., 2013).

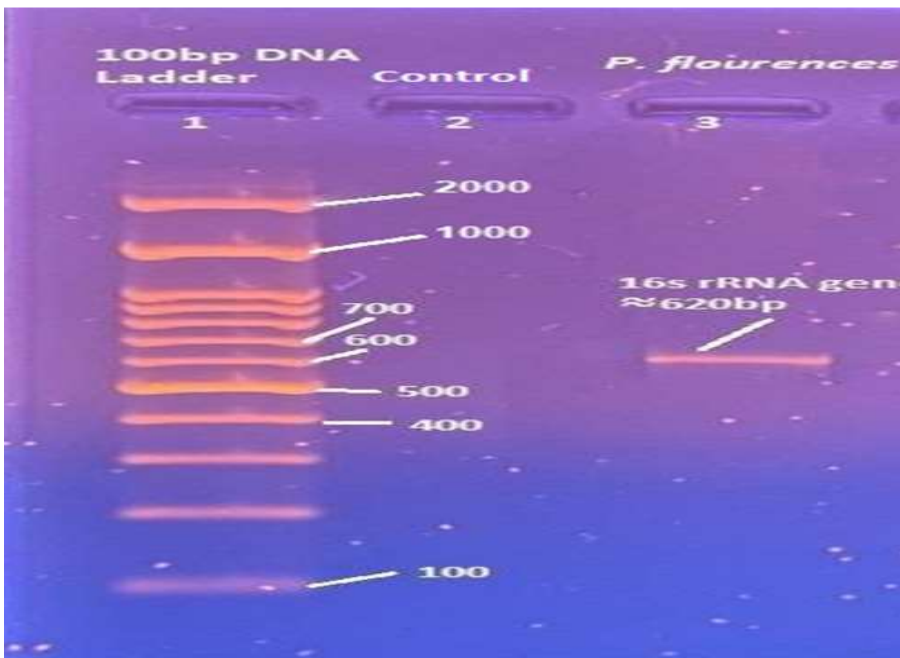

Figure 2 shows the gel for the 16s rRNA gene PCR products. Ethidium bromide and 1% agarose gel are used. Lane 1, 100 bp DNA ladder. Lanes 2 are in negative control. Lane 3 P. fluorescens genomes PCR products.

Plant Growth Promoting Activities

All 18 analyzed Pseudomonas isolates produced IAA in vitro on LB medium supplemented with tryptophan, demonstrating their ability to convert tryptophan into IAA. (Table 5). The pink to red color change of the broth culture indicated the formation of the IAA. Analyses of statistical data revealed that IAA production varied significantly amongst isolates. Among all the isolates, Mpf14 produced the most IAA, followed by Gpf13. These IAA makers could act as plant

growth promoters if tryptophan, an IAA precursor, is available. Adding Fe-H2SO4 solution and IAA to broth culture results in a complex that gives the broth culture its pink color.The good results in colorimetric tests may be caused by the indole that many microorganisms produce. (Ahemad and Kibret, 2013), The least amount of IAA was produced by isolate Wpf5. The amount of IAA chemicals produced in vitro varies, according to observations, depending on the bacterial genus, species, strain, or culture media conditions like aeration and pH. (Radwan et al., 2002). The present study discovered variation in PGPR isolates' ability to produce IAA, which had previously been reported (Zahid, 2015).This difference is also due to the various metabolic pathways and gene positions. Moreover, Activated free IAA can be converted to conjugates by enzymes (Islam et al., 2009). Other scientists discovered high levels of IAA synthesis in bacterium isolates from the genera Pseudomonas spp., Bacillus spp., Rhizobium spp., and Mesorhizobium spp. (Evangelista et al., 2017).

Table 1: The plant growth promoting properties of P. fluorescens

Isolate code	IAA Production	HCN Production	Siderophore production	Quantitative IAA production mg.ml^{-1}	Iron Solubilization (clear zone (%)
Cpf1	++	±	+	20.36^{ij}	15.13^{i}
Cpf2	+	+	++	22.10^{ij}	18.27^{h}
Cpf3	++	+	+	20.19^{hi}	41.92^{a}
Wpf4	+	-	++	18.19^{jk}	23.05^{ef}
Wpf5	+	+	+	12.10^{n}	20.32^{fgh}
Mpf6	±	++	++	37.55^{jk}	26.53^{e}
Mpf7	-	+	+	33.1^{ef}	15.91^{i}
Kpf8	+	++	+	41.09^{cd}	38.68^{b}
Zpf9	+	+	+	15.18^{g}	24.58^{i}
Zpf10	+	+	+	20.56^{h}	29.76^{d}
Spf11	±	-	+	23.80^{f}	22.30^{fg}
Spf12	±	+	+	23.77^{f}	21.98^{f}
Gpf13	++	±	++	49.49^{b}	19.43^{gh}
Mpf14	+++	+++	+++	49.90^{a}	33.87^{c}
Mpf15	+	++	++	20.05^{ij}	10.32^{j}
Dpf16	+	+	+	25.99^{g}	9.80^{j}
Bpf17	+	±	+	18.87^{ij}	14.70^{i}
BPf18	±	++	++	20.50^{h}	21.80^{fg}

Siderophores Production

The fluorescent Pseudomonas isolates have been shown to produce a wide range of secondary metabolites, including siderophores, which have antagonistic capabilities toward a number of phytopathogens. There were 38 isolates total, and 18 isolates were (Cpf1,Cpu2,Cpf3,Wpf4,Wpf5,Mpf6,Mpf7,Kpf8,Zpf9,Zpf10,Spf11,Spf12,Gpf13,Mpf14,Mpf15,Dpf6,Bpf17and BPf18), which belonged to the kings media were positive for siderophores production, as evidenced by the development of a zone that was yellow or orange in color and encircled the bacterial colony. (Table 5). The results showed that the ability of each isolate to create siderophores varied, and that these siderophores were typically antagonistic to pathogenic fungus when produced by P. fluorescens. (Suryakala et al., 2004). Budzikiewicz., 1993 developed that yellow green siderophore producing Pseudomonas species that are responsible for enhanced plant growth. Pseudomonas fluorescens and P. putida produce Pyochelin and Pyoverdin, two different types of siderophores responsible for metal acquisition. (Bultreys et al 2003). Develops Pyoverdins are produced by strains that have similarities in their peptide chains, which are termed siderophors. P. fluorescens produces 18 siderophors. The availability of trace elements like iron, zinc, and other similar elements is another essential direct PGP component. Which are necessary for different metabolic pathways. Siderophores,

which are created by a variety of soil microbes, retain iron in the soil and make it available to plants. (Tokala et al. 2002; Nassar et al. 2003). Despite the fact that siderophores initially seem to only be found in microbes (Neilands 1981, 1995), it is currently reportedly also generated by plants (Romheld and Marschner 1986; Dell'mour et al. 2012). According to the chemical components of the chelating ligands, they are divided into four major groups, catecholates,,hydroxamates, hydroxypyridonates, and aminocarboxylates (Stintzi et al. 2000), but only a few number of siderophores have been described for mixed functional groupings (Hider and Kong 2010). The stability of the complex they create with iron depends on their redox potential and reactivity, which vary. The stability of the complex influences the many functions of siderophores. Because of their high affinity for iron compound, catecholate siderophores are most likely active in the bioleaching of iron from mineral ores. (Kraemer 2004; Rogers and Bennett 2004). Siderophores are also essential for indirectly promoting plant growth because they prevent phytopathogens by depriving them of needed iron. (Ahmed and Holmstrom 2014).

A select number of microorganisms create phytohormones, which are plant growth regulators that have a direct impact on plant growth. (Pattern and Glick 2002; Hayat et al. 2010). These hormones, which stimulate plant growth, include auxins (indole-3-acetic acid), gibberellins, cytokinins, abscisic acid, and ethylene. The synthesis of extracellular enzymes and antimicrobial compounds by PGPRs is one of the indirect PGP mechanisms that allow them to serve as biocontrol agents against diverse plant diseases. (Doumbou et al. 2002; Glick 2012). The main producer of bioactive compounds has been Actinobacteria, specifically the genus Streptomyces. (Alexander 1977) and have demonstrated significant biocontrol activity against many phytopathogens. (Wang et al. 2013). They produce close to 60% of the essential antibiotics for agriculture. (Ilic et al. 2007). It has been noted that Streptomyces griseoviridis is antagonistic to a number of plant diseases, including Fusarium and Rhizoctonia species. (Tahvonen 1982; Tahvonen and Lahdenpera 1988).

Production of Hydrogen Cyanide (HCN)

The fact that the filter paper turned from orange to brown after the incubation period showed that each of the 18 Pseudomonas fluorescens isolates examined had a significant capacity for producing hydrogen cyanide. (Table 5). Numerous rhizobacteria produce hydrogen cyanide, which is thought to be essential for the biological management of plant diseases. (Shaikh et al., 2014). Despite the fact that hydrogen cyanide produced by rhizobacteria on specific hosts can be used as a biological control agent, host plants are not negatively impacted by cyanide's general metabolic inhibitory effects. (Saharan and Nehra, 2011). Some gram-negative bacteria, including P. fluorescens, produce the secondary metabolite cyanide. (Knowles and Bunch 1986). generally, hydrogen cyanide (HCN) is considered as a secondary metabolite with an ecological function that gives the producer strains a selective advantage. HCN is not involved in growing, energy storage, or essential metabolism. (Vining 1990).

By reducing fungal infections, HCN-producing characteristics have a significant impact on plant development. (Aarab et al. 2019). In addition to acting as a biocontrol agent, HCN generated by PGPR also helps in substrate geochemical processes such metal chelation. A large number of biocontrol PGPR can produce hydrogen cyanide (HCN) (Santoyo 2012).

The HCN that PGPR produces appears to function in combination with other biocontrol mechanisms used by the same bacteria. Among other metalloenzymes, HCN inhibits cytochrome c oxidase, which makes it toxic (Nandi et al. 2017). The strain P. fluorescens CHA0 produces hydrogen cyanide, As a result, it gains significant biocontrol potential. hcnABC genes are required for the maximum expression of HCN biosynthesis in P. fluorescens when they are regulated by the anaerobic regulator (ANR) and global activator (GacA). (Blumer and Haas 2000).

Molecular identifications of bacteria and desired genes

Bacteria were identified by molecular identification to confirm their species and strainsIn this study, a universal primer for 16s rRNA was used, and the amplified genes were visualized on an agarose gel. Approximately 620 bp was amplified from each gene, This is similar to the size of Pseudomonas spp.'s 16S rRNA gene. (Figure 3, lanes 3). (Waldeisen JR et al., 2011; Patel et al., 2013). The Sanger technique was used to sequence the amplified genes, and the isolated bacteria's species and strains were confirmed. (https://oligomer.com.tr/). Molecular PCR and agarose gel techniques were used to confirm that both bacteria contained all of the desired genes. According to the results in (Fig 4), the size of the PsiB gene in tested bacteria was approximately 937bp, which was similar to the gene size determined by NCBI, and the negative control gene size was amplified. Furthermore, the amplified size of the PsiG gene in bacteria was around 571bp, and no PCR product size was observed in control treatment (Fig.4). Depending to the results, it can be said that the ability of P.fluresencs in iron solubilizing due to the siderophore formation

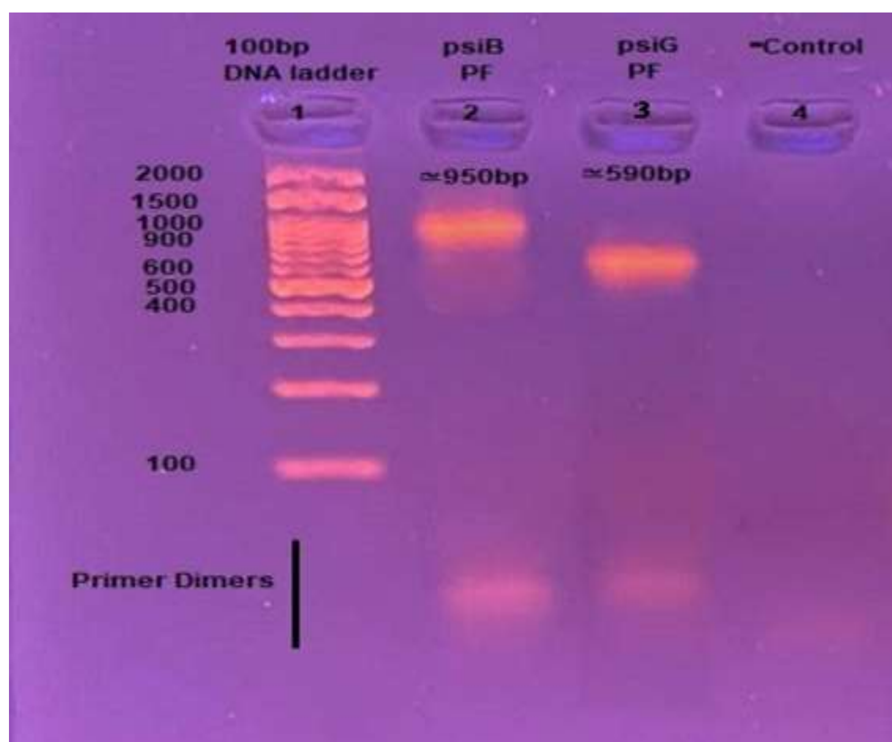

Figure 3 shows the gel for the PCR-produced copies of the psiB and psiG genes . Ethidium bromide and 1.5% "(w/v)" agarose gel are used. Lane 1, 100bp DNA Ladder . psiB and psiG that have been amplified are found in lanes 2 and 3, respectively. Negative control in lane four.

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