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SCIENTIFIC JOURNAL
OF MEDICAL RESEARCH

Vol. 2, Issue 7, pp 135-146, Summer 2018

ISSN: 2520-5234

ORIGINAL ARTICLE

# Phenetic and Phylogenetic Analysis of *Kocuria palustris* and *Kocuria rhizophila* Strains isolated from Healthy and Thalassemia Persons

**Mohsen A. A. Al Bayatee** [1] and **Essra Gh. Alsammak** [2]

[1] Department of General Science, College of Basic Education, University of Mosul, Mosul, Iraq.
[2] Department of Biology, College of Science, University of Mosul, Mosul, Iraq.

**ARTICLE INFORMATIONS**

**Article History:**
Submitted: 26 July 2018
Revised version received:
14 August 2018
Accepted: 16 August 2018
Published online: 1 September 2018

**Key words:**
*Kocuria palustris*
*K. rhizophila*
16S rRNA Gene Sequence
Whole cell protein bands profile

**Corresponding author:**
Mohsen A. A. Al Bayatee
Email: mohsen3d89@yahoo.com
Department of General Science
College of Basic Education
University of Mosul
Mosul
Iraq

**ABSTRACT**

**Objective:** The normal habitat of *Kocuria palustris* and *K. rhizophila* include mammalian skin, soil and rhizoplane. The aim of this study is to find the relatedness among several strains of *K. palustris* and *K. rhizophila* isolated from healthy and thalassemia patients according to the biochemical tests, make phylogenetic analysis to construct a database for whole cell protein band profile of these species.

**Methods:** Ninety samples were collected from healthy and thalassemia patients skin in April of 2013, seventeen samples were revealed bacterial isolates. The diagnosis was performed using conventional biochemical tests , eight of them analyzed according to their 16S rRNA gene sequence and used as reference to confirm the diagnosis of other isolates depending on phenetic and protein bands clustering patterns. Three different software used , IBM SPSS v.19, MEGA 5.22 and CLIQS v.1 in dendrogram building and interpreting the results.

**Results:** Seventeen strains of the genus Kocuria were isolated from 90 sample collected from human skin. These strains appeared as coccoid Gram positive cells and had smooth yellow colonies, slightly convex. Three strains were related to *K. palustris* and fourteen strains were related to *K. rhizophila*. The morphological dendrogram built upon 76 phenetic characters, and divided to three clusters with similarity ratio of 94-99%. Eight strains were analyzed for their 16S ribosomal RNA gene sequences and compared with NCBI by using BLAST which gave 94-97 % similarity with reference strains. The whole cell protein profile was analyzed by CLIQS v.1, similarity ratio ranged from 29-100% among strains in dendrogram. Analysis of amino acids composition in cell wall using paper chromatography showed four amino acids, alanine, glutamic acid, glycine and lysine.

**Conclusion:** The study showed good relatedness among strains of *K. palustris* and *K. rhizophila* according to morphological and biochemical tests and phylogenetic analysis, but low similarity according to whole cell protein bands profile.



**Citation: Al Bayatee M.A.A.** and **Alsammak E.Gh.** "Phenetic and Phylogenetic Analysis of *Kocuria palustris* and *Kocuria rhizophila* Strains isolated from Healthy and Thalassemia Persons". Sci. J. Med. Res. 2018, 2 (7): 135-146.

## INTRODUCTION

The genus Kocuria named after Miroslav Kocur, a Slovakian microbiologist and characterized by Stackebrandt in 1995 [1], as new genus from micrococcus according to phylogenetic studies by using 16S rRNA gene sequence [2], the genus Kocuria related to phylum Actinobacteria, class Actinobacteria, order Actinomycetales, suborder Micrococcineae, family Micrococcaceae[2]. This genus differentiated from Micrococcus by phylogenetic and chemotaxonomic studies. Kocuria strains can be isolated from different

sources include plants, animals, soil, air and fermented foods [3,4] . Until this time there are more than 18 species related to Kocuria and characterized by Gram positive strains, coccoid cell shaped and cells arranged in diploids, tetrads, short chains, cubical packets of eight cells and irregular clusters, non-motile, non-endospore forming, and can be differentiated from other genera within Actinobacteria on the bases of peptidoglycan type(L-Lysine $_{3/4}$), the presence of galactoseamine, glucosamine as main amino sugars in the cell wall, DNA G+C mol is 66-75%, most of recorded strains were mesophilic [3, 5].

Some studies showed that five species may cause opportunistic infection in immunocompromised patients, like *K. roseae* which cause nephritis, peritonitis in diabetes patients [6,7,8], while *K. kristinae* may be responsible of infections associated with catheter usage, in pregnant and as a causative agent of cholecystitis [5,7,8]. No research referred to *K. palustris* or *K. rhizophila* as causative agent for human disease but may present on human skin as normal flora.

The aim of this study is to isolate and diagnose of *K. palustris* and *K. rhizophila* from healthy and thalassemia patients, and to find the relatedness among the isolates according to their biochemical tests, make phylogenetic analysis to construct a database for whole cell protein band profile of these species.

## MATERIALS AND METHODS

### Sample collection

Samples were collected from human skin by using wet sterilized swabs, including thalassemia patients (from Ibn Al Athir Teaching Hospital for thalassemia) and health persons in April of 2013, cultured on nutrient agar containing 5.5% sodium chloride, incubated for 24-72 hours at 35°C.

### Morphological characterization

Characterization was done by identifying colony morphology including size, margin, height, color and gram staining, cells shape, arrangement, motility [9], Rod-Coccus cycle to differentiate Kocuria from Arthrobacter an Rhodococcus [10].

### Biochemical tests

The strains differentiated from *Staphylococcus* spp. by sensitivity tests to bacitracin and furazolidon using disc diffusion (Kirby-Bauer) method on Mueller Hinton agar. Catalase, oxidase, glucose fermentation, coagulase test, acid production from carbohydrates, aesculin hydrolysis, hemolysis, methyl red, voges proskauer, indole production, nitrate reduction [10], urease test [11], gelatinase test [12], citrate utilization [13], starch hydrolysis and the ability to grow on mannitol salt agar, thioglycolat agar, MacConkey agar, eosin methylene blue agar [14], casein hydrolysis and DNase test [15], Amino acid utilization [16], ability to grow in the presence of potassium cyanide [17], sodium chloride tolerance [18], antimicrobial sensitivity test [19, 20] (Culture media were supplied by oxoid). The phenetic dendrogram was built by using IBM SPSS v.19 software by nearest neighbor with simple matching coefficient.

### DNA Extraction

**Cell extract was prepared as following:** 70 milligrams of bacterial cells were collected from colony aged 36 hours at 35°C, placed in Eppendorf tubes (1.5ml). The cells washed twice with sterilized normal saline to remove the undesired traces of medium or extracellular proteins [21].

The washed cells treated with EDTA-Lysozyme solution, 10mg/ml at pH 6.6, 37°C for two hours [22]. Then the cell extract treated with equal amount of SDS-Solubilization Buffer (Table 1) for 5 min at 95°C. To inhibit the enzymatic activity, the suspension was cooled at room temperature and stored under -20°C until use.

Table 1: **SDS-Solubilization Buffer compositions (according to Feligini Et al.)[23].**

| Component | Concentration / quantity |
|---|---|
| Tris HCl | 10 mM , pH 7.5 |
| EDTA | 1 mM |
| NaCl | 51 mM |
| SDS Sodium Dodecyl Sulfate | 2 mg/ml of solution |

**DNA purification and quantification:** The DNA was purified according to Nishiguchi *et al.* [24]. The DNA concentration and purity was detected by nanodrop spectrophotometer (Biodrop, England) at wave length 280/260 nm.

**16S rRNA Gene Amplification ( Polymerase Chain Reaction):** This reaction done using forward primer 27f and reverse primer 1329r supplied by Promega company at final concentration 10 pmol, as shown in Table 2.

Table 2: **Primer sequences used for amplification of 16S rRNA gene, according to Lane [25].**

| Gene size base pair | Primer type | Primer Sequence 5´to 3´ |
|---|---|---|
| 16S rRNA | 27f upstream | AGAGTTTGATCTTGGCTCAG |
| | 1329r downstream | GACGGGCGGTGTAC |

The mixture was prepared at final volume 50 µl for each sample using *Go Taq* Green Master Mix ( supplied by Promega comppany) as the program shown in Table 3, then the amplified gene was electrophoresed on 1% agarose gel beside of DNA ladder 100 bp. Under 60 volts, 100 milliampere, 6 watts for 75 min. The gel was stained with ethidium bromide solution for 1 hour and photographed on UV transilluminator.

Table 3: **Amplification program of 16S rRNA gene using MultiGene Optimax Thermal Cycler [25].**

| Steps | Temperature °C | Time/period | Cycle No. |
|---|---|---|---|
| First denaturation period | 94 | 5 min. | 1 |
| Denaturation period | 94 | 35 sec. | |
| Annealing period | 58 | 1.35 min. | 35 |
| Elongation period | 72 | 1.35 min. | |
| Last annealing period | 72 | 10 min. | 1 |

**16S rRNA gene sequence analysis:** The 16s rRNA gene analyzed by Microgen Laboratory in United State of America. The sequences of each sample were compared with the same gene sequences at National Center Biotechnology Information (NCBI) at (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) to find nearest strain to the tested strains. The phylogenetic dendrogram was built using Molecular Evolutionary Genetic Analysis software (Mega) v. 5.22.

**The whole cell protein profile study**

**Whole cell protein extraction:** The cell extraction prepared according to De *et al* [23]. Then purified from DNA and carbohydrates by phenol: chloroform: isoamyl alcohol mixture. The med-layer (isoamyl-protein) collected in eppendorf tube and stored under -20°C without further purification.

**Protein quantification:** Achieved by Bradford method[27] using UV1800 Shimadzu spectrophotometer, the final concentration of protein adjusted to 5µg/ml by sterilized distilled water.

**Sodium dodecyl sulphate polyacrylamide gel (SDS-PAG) preparation:** The discontinuous gel consisted of stacking gel 5% and resolving gel 12% depending on Manufacture Lab net. Inc. In Enduro$^{TM}$VE10 vertical gel system.

**Protein electrophoresis:** The samples of protein were treated with equal volume of 2X sample buffer (Table 4) for 5 min. At 100°C [28,29], 10 µl of each sample loaded on the gel, then electrophoresed along with broad range protein molecular marker (from Promega comp.) in 1X tris-glycine tank buffer SDS, under 100 volts, 0.04 ampere, 4 watts about 3 hours until the smallest band reaches 1cm from the bottom of the gel.

Table 4: **Compositions of 2X Sample Buffer.**

| Component | Quantity |
|---|---|
| Glycerol 50% | 2ml |
| β-mercaptoethanol | 0.5 ml |
| Sodium dodecyl sulphate solution 10% | 4ml |
| Tris-HCl 5M | 2.5 ml |
| Bromophenol Blue Solution 1% | 1ml |
| Distilled Water | To final volume 10 ml |
| All contents dissolved , divided in Eppendorf tubes 1.5ml and stored under -20°C. | |

**Fixing, Staining and destaining of the gel**

Each gel fixed in fixing solution composed of (glacial acetic acid: methanol: distilled water at 10: 50: 40 volume ratio, respectively ) for 2-4 hours, the fixing solution removed and the gel stained with coomassie brilliant blue R250 supplied by Bioworld Comp. at concentration 0.125gm/100ml of fresh fixing solution, leaved for 24 hours with shaking each hour. The gel washed 2-3 times with washing solution (the same as fixing solution) until the bands observed clearly. The gel photographed by BenQ GH700 camera (BenQ corporation).

**Protein band profile analysis:**The gel photos (images) analyzed by Core Laboratory Image Quantification Software (CLIQS) v.1 from total lab, in the presence of broad range protein molecular marker, and the molecular weight were obtained for each band in the gel, the relationship among the strains was built as dendrogram using UPGMA with pearson correlation factor.

**Cell wall amino acids analysis by paper chromatography**

The cell walls prepared as described by Baboolal [30], lyophilized and stored under -20°C until used.

The amino acids of cell wall analyzed on cellulose paper[31], by dissolving 3mg of cell wall(dry weight) in 1ml 6N HCl in tightly closed screw capped eppendorf tubes, treated under 100°C for 18 hours, the extract filtered through filter paper 1250- B, the filtered solution dried on boiling water, dissolved again in 1 ml of distilled water and dried. The remaining precipitate dissolved in 0.25ml distilled water, 2µl. of this solution spotted on base line of chromatograph paper, along with 0.8µl of 2 mg/ml of standard amino acids (alanine, glutamic acid, glycine, lysine and diaminopimelic acid) supported by sigma aldrich.

The solvent system used for separation of amino acids composed of methanol: distilled water: 6N HCl: pyridine in ratio 80: 26: 10: 4 repetitively, for 3.5 hours in thin layer chromatography glass tank. The excess solvent removed from chromatographed paper and dried with hot air in fumed hood, stained with ninhydrin solution (0.2 mg/100 ml acetone) then dried at room temperature and developed in oven 100°C for 3min. To appear as purple or blue-purple colored spots. To get the Rf of each amino acid, the distance of amino acid from base line, divided on the distance of solvent from base line. Comparing the Rf of unknown with standard amino acids.

## RESULTS AND DISCUSSIION

The strains of Kocuria separated from *Staphylococcus* spp. Through sensitivity test to bacitracin and furazolidon and glucose fermentation . Kocuria species were sensitive to bacitracin, resistant to furazolidon and not ferment the glucose [10, 32]. The strains of *K. palustris* separated from strains of *K. rhizophila* depending on their biochemical tests [33].

Three strains of *K. palustris*, fourteen strains of *K. rhizophila* were obtained from nineteen sample collected from healthy persons and thalassemia patients' skin. The largest ratio of isolation for *K. rhizophila* was 25% from healthy person skin samples, then 12.8% from thalassemia patients samples. Also, the *K. palustris* isolation ratio was10% from healthy persons, 1.4% from thalassemia patients as shown in the Table 5.

**Characterization of bacterial isolates**

**Colony morphology :** *K. palustris* characterized by pale-yellow smooth circular slightly convex colonies with entire edges about 0.9 mm in diameter of 24 hours aged colony on nutrient agar (37°C).

*K. rhizophila* had yellow smooth circular slightly convex colonies with entire edges,about 1mm in diameter of 24 hours aged colony on nutrient agar 37°C.

**Microscopic examination:** Gram positive coccoid cells in diploids, tetrads, irregular clusters, non-motile non endospore – forming bacteria. The diameter of *K. palustris* cells was 1.3-1.4 microns, whereas *K. rhizophila* cells' diameter was 1.3-1.4 microns.

**Biochemical tests identification:** Biochemical identification and phenetic dendrogram building depended on 76 characters (Table 6), the strains were identified as *K. palustris* and *K. rhizophila*, the phenetic dendrogram was built by using single linkage method and simple matching coefficient $S_{sm}$, the strains clustered in three clusters as shown in Figure1.

Table 5: **Samples number , isolation ratio for *K. palustris* and *K. rhizophila*.**

| Isolation source | Samples No. | *K. palustris* | | *K. rhizophila* | |
|---|---|---|---|---|---|
| | | Strains No. | Isolation % | Strains No. | Isolation % |
| **Thalassemia patients** | 70 | 1 | 1.4 | 9 | 12.8 |
| **Healthy persons** | 20 | 2 | 10.0 | 5 | 25.0 |
| **Sum** | 90 | 3 | | 14 | |

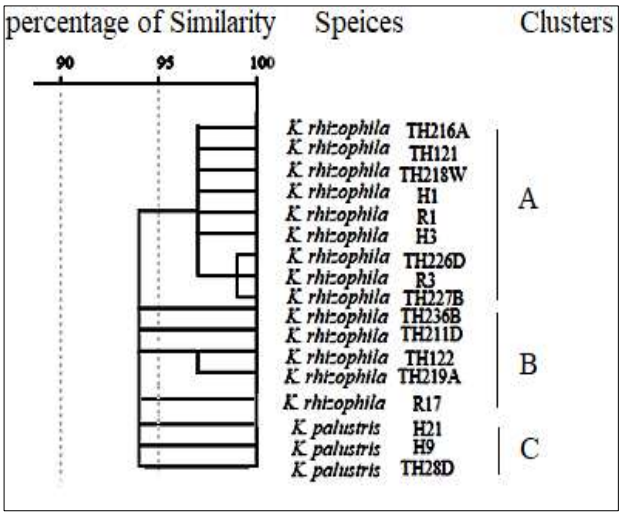



Figure 1: **Phenetic dendrogram for strains related to *K. palustris* and *K. rhizophila* built by single linkage method and nearest neighbor joining with simple matching coefficient.**

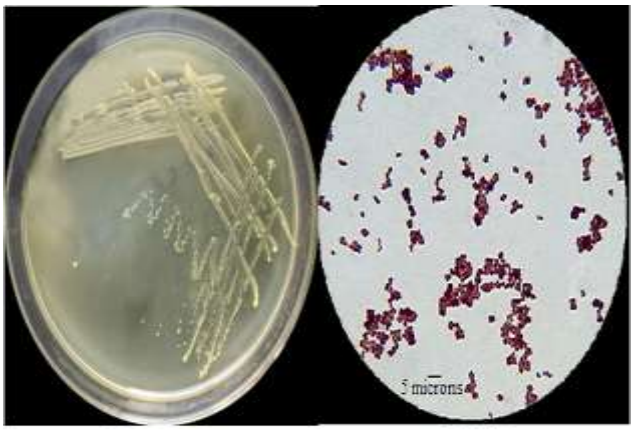
Table 6: **Morphological and Biochemical tests of three clusters.**

| No. | Character | | Cluster A | Cluster B | Cluster C |
|---|---|---|---|---|---|
| 1 | Colony color on nutrient agar | Yellow | v | v | - |
| | | Light yellow | v | v | - |
| | | Pale yellow | - | - | + |
| 2 | Colony size after 24h./37°C | <1mm | v | v | v |
| | | 1-1.5mm | v | v | v |
| 3 | Colony surface | Smooth | v | v | + |
| | | Rough | v | v | - |
| 4 | Colony nature | Creamy | v | v | + |
| | | Mucoid or slime | - | - | - |
| | | Dry | v | v | - |
| 5 | Colony height | Plane | - | - | - |
| | | Raised | - | - | - |
| | | Convex | + | + | + |
| 6 | Colony edges | Entire | + | + | + |
| | | Irregular | - | - | - |
| 7 | Gram positive coccus | | + | + | + |
| 8 | Cell arrangement | Diploid | + | + | + |
| | | Tetrad | + | + | + |
| | | Irregular cluster | + | + | + |
| | | Short chains | - | - | - |
| 9 | Cell size | < 1 Micron | - | - | - |
| | | > 1micron | + | + | + |
| 10 | Growth at | 5°C | - | - | - |
| 11 | | 25°C | + | + | + |
| 12 | | 35°C | + | + | + |
| 13 | Enzyme production | Oxidase | - | - | - |
| 14 | | Catalase | + | + | + |
| 15 | | Urease | v | v | v |
| 16 | | DNase | - | - | - |
| 17 | | Coagulase | - | - | - |
| 18 | Hemolysis (Blood hydrolysis) | | - | - | - |
| 19 | Acid production | Glucose | v | v | v |
| 20 | aerobically from | Lactose | - | - | - |
| 21 | | Maltose | v | v | + |
| 22 | | Mannitol | - | - | - |
| 23 | | Sucrose | v | v | v |
| 24 | | Galactose | - | - | - |
| 25 | | Sorbitol | - | - | - |
| 26 | | Arabinose | - | - | v |
| 27 | | Melibiose | - | - | - |
| 28 | Nitrate reduction | | v | v | + |
| 29 | Hydrolysis of | Starch | v | - | - |

| | | | | | |
|---|---|---|---|---|---|
| 30 | | Aesculin | - | - | v |
| 31 | | Casein | v | v | - |
| 32 | | Gelatin | v | v | v |
| 33 | Citrate utilization | | v | v | v |
| 34 | Growth on | Potassium cyanide containing nutrient broth | v | v | v |
| 35 | | Simon citrate agar | v | v | v |
| 36 | | Thioglycolate agar | - | - | - |
| 37 | | Eosin methylene blue agar | - | - | - |
| 38 | | MacConkey agar | - | - | - |
| 39 | | Mannitol salt agar | + | v | + |
| 40 | | Tryptic soy agar | + | + | + |
| 41 | | Tryptic soy agar + 5.5% NaCl | + | + | + |
| 42 | | Tryptic soy agar + 7.5 % NaCl | + | v | + |
| 43 | | Tryptic soy agar + 9.5 % NaCl | + | v | + |
| 44 | Utilization of amino | L-arginine | v | v | v |
| 45 | acids | D-alanine | v | v | - |
| 46 | | L-alanine | v | v | - |
| 47 | | Phenyl alanine | - | v | v |
| 48 | | Tryptophan | - | - | - |
| 49 | | Isoleucine | v | - | - |
| 50 | Methyl red test | | v | - | - |
| 51 | Voges proskaur test | | - | - | - |
| 52 | Indol production | | - | - | - |
| 53 | Motility | | - | - | - |
| 54 | Sensitivity to | Bacitracin 10 µg/disc | + | + | + |
| 55 | antibiotics | Furazolidon 100 µg/disc | - | - | - |
| 56 | | Ampicillin 25 µg/disc | v | v | v |
| 57 | | Chloramphenicol 10µg/disc | + | + | v |
| 58 | | Cefixim 5 µg/disc | - | - | - |
| 59 | | Ciprofloxacin 10 µg/disc | + | + | + |
| 60 | | Gentamycin 10 µg/disc | + | + | + |
| 61 | | Clindamycin 10 µg/disc | + | + | + |
| 62 | | Erythromycin 15 µg/disc | v | v | v |
| 63 | | Nitrufurantoin 100 µg/disc | - | - | - |
| 64 | | Imepinim 10 µg/disc | + | + | + |
| 65 | | Lincomycin 10 µg/disc | + | + | + |
| 66 | | Methicillin 10 µg/disc | - | - | - |
| 67 | | Neomycin 30 µg/disc | + | + | + |
| 68 | | Naldixic acid 30 µg/disc | - | - | - |
| 69 | | Penicillin 10 µg/disc | + | + | + |
| 70 | | Pepracillin 30 µg/disc | v | v | + |
| 71 | | Carbincillin 25 µg/disc | - | - | - |
| 72 | | Refamycin 10 µg/disc | + | v | + |
| 73 | | Streptomycin 25 µg/disc | v | v | + |
| 74 | | Tetracycline 10 µg/disc | + | + | + |
| 75 | | Trimethoprim 10 µg/disc | v | v | + |
| 76 | | Vancomycin 10 µg/disc | + | + | + |

+ : positive , sensitive to antibiotic - : negative, resistance to antibiotic v : variable

**First Cluster A:** Consisted of nine strains of *K. rhizophila* clustered together at 97% of similarity ratio, and characterized by bright yellow to pale yellow, smooth, slightly raised circular colonies, coccoid cells about 1.3 – 1.6 µm in diameter, arranged in diploids, tetrads and irregular clusters as shown in Figure 2. All of them were positive for catalase, not motile, non-endospore forming bacteria, grew at 25°C and 35°C, but not at 5°C, other characters were shown in Table 6. Most of these results were similar to that shown by Kovács [33] about *K. rhizophila*.




A B

Figure 2: **A, colonies of *K. rhizophila* R1 on nutrient agar. B, cell Shsape of *K. rhizophila* R1 under microscope, 100X.**

**Second Cluster B:** Consisted of five strains of *K. rhizophila* clustered together at 94 % similarity ratio, and characterized by yellow smooth creamy circular colonies. The cells were coccoid shape ranged from 1.4-1.6 µm in diameter, arranged in diploids and irregular clusters as shown in Figure 2, other characters were shown in Table 6. Most of these characters similar to that shown by Kovács [33].

**Third Cluster C:** Included three strains of *K. palustris* clustered together at 94% similarity ratio. They characterized by light yellow- pale yellow, circular colonies. The cells were gram positive cocci, 1.3-1.4µm in diameter, arranged in diploids, tetrads and irregular clusters as shown in Figure 3, other characters were shown in Table 6. Almost their characters similar to that shown by Kovács [33].


A B

Figure 3: **A, Colonies of *K. palustris* H21on nutrient agar . B, Cells shape of *K. palustris* H21 under microscope ,100X.**

**Identification of strains according to 16S rRNA gene.**

After 16S rRNA gene amplification using forward primer 27f, and reverse primer 1329r, as shown in Figure 4, the gene size ranged between 1380-1450 bp. by using CLIQS v.1 software.

Genes for eight strains were selected, and sent to Microgen Laboratory/ USA for 16S rRNA gene sequencing, using an ABI 3730 XL DNA analyzer. The sequences analyzed at NCBI and using BLASt, the similarity ratio ranged between 94-97% as shown in Table 7.

The sequences analyzed with Mega 5.22 software by clustal W, the similarity ratio and phylogenetic dendrogram built using nearest neighbor single linkage method [34, 35, 36, 37, 38], which was the best statistical method in phylogenetic dendrogram building[39]. The eight strains divided in to four clusters with similarity ratio 86.4-99.8 % as shown in Table 7 and Figure 5. The phylogenetic clustering agreed with phenetic clustering for strains related to *K. palustris*, while there was slight difference between phylogenetic and phenetic clustering for strains related to *K. rhizophila* with close similarity in two clustering methods.

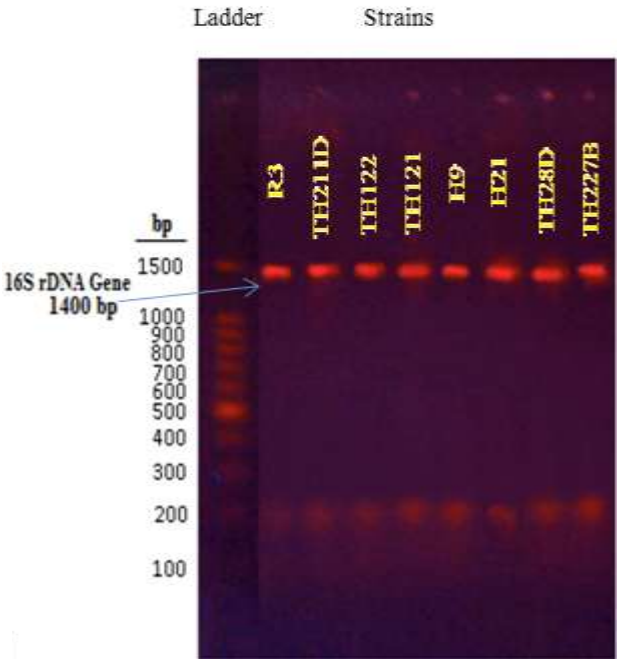



Figure 4: **Results of 16S rRNA gene for selected strains after electrophoresed on 1% agarose gel.**

Table 7: **Identified strains comparing to reference strains in NCBI , similarity ratio and gene length.**

| Strains | Reference Strains at NCBI | Gene length base pairs (bp.) | Similarity ratio % |
|---|---|---|---|
| *K. rhizophila* R3 | *K. rhizophila* Strain XFB-BG | 1269 | 96 |
| *K. rhizophila* TH211D | *K. rhizophila* Strain XFB-AX | 1271 | 96 |
| *K. rhizophila* TH122 | *K. rhizophila* Strain 236-4A | 1235 | 94 |
| *K. rhizophila* TH121 | *K. rhizophila* Strain R-42745 | 1282 | 97 |
| *K. palustris* H9 | *K. palustris* Strain JPR-01 | 1248 | 96 |
| *K. palustris* H21 | *K. palustris* Isolate PDD-31b-3 | 1277 | 97 |
| *K. palustris* TH28D | *K. palustris* Strain IARI-ABL-32 | 1260 | 97 |
| *K. rhizophila* TH227B | *K. rhizophila* strain TA68 | 1255 | 96 |

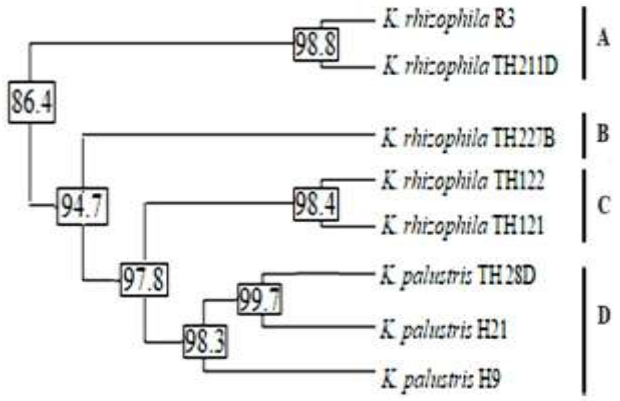



Figure 5**: Phylogenetic dendrogram for some strains belong to *K. rhizophila* and *K. palustris* based on 16S rRNA similarity ratio using Mega 5.22 Software.**

**First cluster A:** Included two strains R3 and TH211D related to *K. rhizophila* , clustered together at 98.8% similarity ratio as shown in Figure 5, they also clustered in phenetic dendrogram at 94% similarity ratio as shown in Figure 1.

**Second cluster B:** Contained one strain TH227B, related to *K. rhizophila*, which grouped with cluster A at 86.4 % similarity ratio, and 94.0% similarity ratio with the third and fourth cluster in phylogenetic dendrogram. This distance from other strains of *K. rhizophila* may be due to changing or modification in some nucleotides within 16S rRNA gene resulted from mutation in this gene. It clustered with *K. rhizophila* R3 strain within phenetic dendrogram at 99% similarity ratio as shown in Figure 1, which confirmed it's relatedness with *K. rhizophila*.

**Third Cluster C:** Included two strains TH122 and TH121of *K. rhizophila*, bind together at 98.4% similarity ratio.

**Fourth Cluster D:** Consisted of three strains of *K. palustris* presented by H9, H21 and TH28D strains. grouped together at 98.3% similarity ratio, which agreed with phenetic clustering as shown in Figure 1 at 94 % similarity.

It's known that 16S rRNA gene contains highly conserved regions, rarely nucleotide changed within closely related Bacteria. Therefore the similarity ratio larger than 99% correlate to species level, while less than 99% refer to genus level and sometimes the ratio 93% - less than 97 % may refer to new genus or new species and needed further investigations to confirm that[40].

**Dendrogram building according to whole cell protein bands profile**

Eighty six protein bands were detected and analyzed using CLIQS v.1 software to obtain band positions, the protein dendrogram was obtained by using Unweighted Pair Group Method Using Arithmetic Average (UPGMA)and pearson correlation factor, which resulted in protein band dendrogram [41, 42, 43, 44, 45, 46, 47, 48, 49] that divided into eight cluster as shown in Figure 5.

**First cluster A:** Included one strain TH218W of *K. rhizophila* which related to the second cluster B at 38.5% similarity ratio and shared by four bands of protein.

**Second cluster B:** Consisted of two strains H9 and H21 of *K. palustris* at 56% similarity ratio and participated in eleven protein bands.

**Third cluster C:** Contained one strain TH28D of *K. palustris* and correlated with second cluster B at 48% similarity ratio and participated in eight bands. Also correlated with second cluster strains according to phenetic dendrogram at 94% similarity ratio as shown in Figure 1, and according to phylogenetic dendrogram, were correlated together at 98.3% similarity ratio as shown in Figure 5, which ensure the identification of the strain TH28D that belong to *K. palustris*.

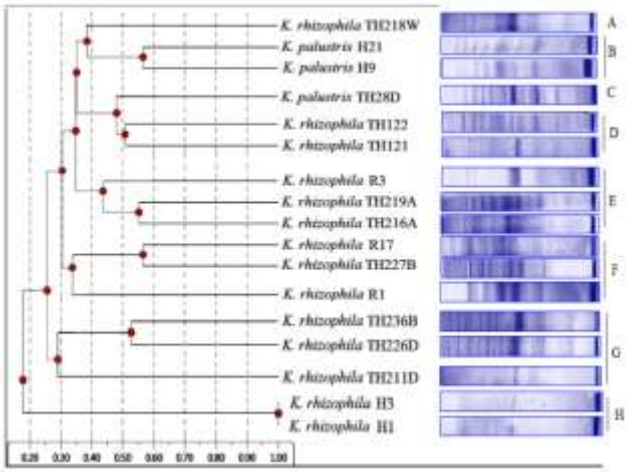



Figure 6: **Whole cell protein profile of some strains of *K. palustris* and *K. rhizophila* using UPGMA and pearson correlation factor by CLIQS v.1 software.**

**Fourth Cluster D:** Included two strains TH121 and TH122 of *K. rhizophila*, correlated together at 51% similarity ratio and participated with them by sixteen bands. The cluster very close to phenetic clustering which gave 94% similarity ratio as shown in Figure 1, and to phylogenetic clustering with 98.4 % similarity ratio as shown in Figure 5.

**Fifth Cluster E:** Consisted of three strains R3, TH216A and TH219A of *K. rhizophila* which correlated together at 44% similarity and participated in five bands. The strains TH216A and TH219A were more closely related at 55% similarity and shared eleven bands as shown in Figure 6, also the two strains TH216A and TH219A clustered at 94% similarity in phenetic dendrogram.

**Sixth Cluster F:** Included three strains R1, R17 and TH227B of *K. rhizophila* clustered at 33% similarity ratio and shared five bands, while the strains TH227B and R17 were more closely related at 56% similarity ratio and shared thirteen bands as shown in Table 8. TH227B and R17 strains correlated together at 97% similarity according to phenetic dendrogram.

**Seventh cluster G:** Consisted of three strains TH211D, TH226D and TH236B of *K. rhizophila* at similarity ratio 29 % which seems to be very low correlation among them, but they correlated according to phenetic dendrogram at 94% similarity. The TH226D and TH236A strains were more closely related with 53% similarity ratio and shared by ten protein bands as shown in Table 8.

**Eighth Cluster H:** It represented the furthest cluster in this dendrogram with least number of bands. Included two strains H1 and H3 of *K. rhizophila* with similarity ratio 100% and shared together by eight bands. This distance may be due to few detected bands for each strain on gel, which did not exceed eight bands, and this due to weakly staining of the bands or destaining taken more time than needed. The two strains correlated together at 97% upon phenetic dendrogram. As shown in Figure 1.

**Explanation of protein bands dendrogram**

This clustering limited to species level, as shown by Villani [50] about *Leuconostoc mesentroids.* Though, some researchers indicated that can be dependent on protein profile for clustering to subspecies level, when the standard conditions present. Some researchers dependent on limited number of protein bands on gel [51, 52, 53, 54], which were more close to each other for comparison and dendrogram building. There are many factors can affect on the SDS-PAGE profile and make difference in the results, like strains nature, genetic variability within same species. In this research strains collected from different individuals that have different nutritional and environmental habitat, some of them suffering from thalassemia who take drugs that can affect on the normal flora physiology, therefore the similarity ratio were med or low. These results close to that obtained by Soomro and Masud [55], Samelis *et al.* results about *Lactobacillus* spp.[56], which explained it as variability of isolation resources. Angelis [57] and Vandamme [58] indicated the same fact or reasons. Priest and Austin [59] indicated difficulty in controlling the environmental conditions when the strains have different physiological requirements, and some proteins may be existed in trace quantities, so they cannot be seen on gel when electrophoresed on gel and stained [60, 61], some proteins may give dense bands which interfere with other bands and cause large effect on the results and give low similarity ratio [62, 63]. There are technical reasons may affect the results, like comparing bands profiles from two separated gels [47] because of different Rf values obtained in each gel even all conditions are the same in two gels electrophoresis. To avoid like this effects, Jackman and Pelczynska [64] indicated normalization (differences correction) to reduce the variability among bands on two separated gels. It can be depend upon standard proteins or molecular weight of known protein bands as references for correction [65, 66, 67]. Other technical effects may be due to staining or destaining periods [64] especially when the proteins existed in trace amounts because coomassie brilliant blue sensitivity threshold is 200-400ng/0.5cm of gel [68].

The molecular weights were comparable in weight, therefore it requires another methods for separation like mass spectrophotometer to confirm the protein dependent classification [69].

**Comparison among the dendrogram building methods**

The best method of classification was 16S rRNA gene sequencing analysis which deals with more than 1312bp. Concerning the phenetic classification gave good results because it depends upon 76 characters. Whereas, protein band profile depends upon constant number of protein bands which ranges between 8-32 bands. Thus the later method doesn't free of defect [66].

**Cell wall composition of amino acids**

The results appeared that all analyzed samples had four amino acids in their cell wall structure, alanine, lysine, glutamic acid and glycine but not diaminopimelic acid (DAP) according to spots appeared on chromatographed paper after staining with ninhydrin. Compared with standard amino acids, these results agreed with that indicated by Cummins and Harris [70], Perkins and Rogers [71], Perkins [72].

The amino acids differed in their Rf within paper, the alanine had Rf value 0.74-0.80, glutamic acid Rf value ranged between 0.66-0.73, lysine Rf value 0.63-0.67, glycine Rf value was 0.55-0.60 while DAP Rf value was 0.36-0.41 as shown in Table 9 , most amino acids appeared as blue - purple colored spots, but DAP appeared as gray yellowish spots as shown in Figure 7.

There were unknown spots far of base line of chromatographed paper had purple to pink color, this might belong to isoleucine or glucosamine which can react with ninhydrin to give like this color [71]. The largest quantity of amino acids in cell walls was alanine because it had dense colored spots comparing with other spots, and this due to presence of alanine in two forms D and L form within cell wall and interpeptide bridges in peptidoglycan [72]. Other amino acids appeared approximately at the same quantity.

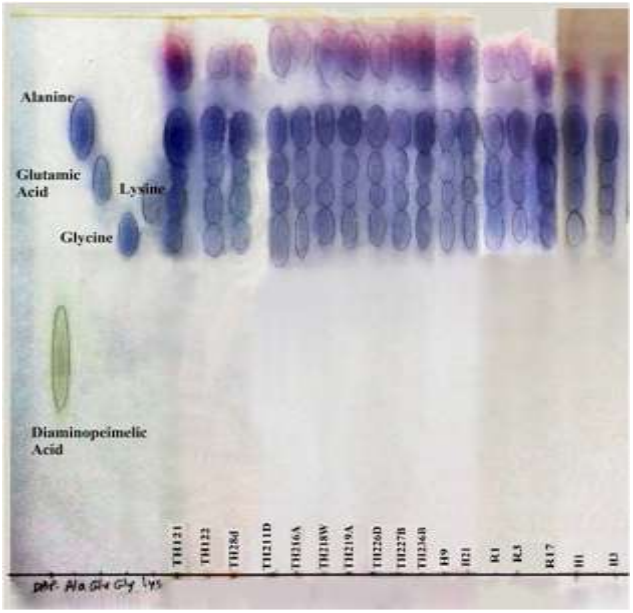



Figure 7: **Cell walls amino acids of *K. palustris* and *K. rhizophila* strains using paper chromatography.**

Table 8: **Protein bands profiles' similarity ratio in *K. palustris* and *K. rhizophila* strains.**

| Cluster | Species | Strain code | Strains No. | Similarity Ratio % | Shared Bands |
|---|---|---|---|---|---|
| A | *K. rhizophila* | TH218W | 1 | 38.5 | 4 |
| B | *K. palustris* | H21, H9 | 2 | 56 | 11 |
| C | *K. palustris* | TH28D | 1 | 48 | 8 |
| D | *K. rhizophila* | TH122 , TH121 | 2 | 51 | 16 |
| E | *K. rhizophila* | R3 | 1 | 44 | 5 |
| | *K. rhizophila* | TH219A, TH216A | 2 | 55 | 11 |
| F | *K. rhizophila* | R17, TH227B | 2 | 56 | 13 |
| | *K. rhizophila* | R1 | 1 | 33 | 5 |
| G | *K. rhizophila* | TH236B, TH226D | 2 | 53 | 10 |
| | *K. rhizophila* | TH211D | 1 | 29 | 3 |
| H | *K. rhizophila* | H3, H1 | 2 | 100 | 8 |

Table 9: **Rate of flow (Rf) of cell walls amino acids of *K. palustris* and *K. rhizophila* strains comparing with standard amino acids Rf.**

| No. | Bacterial isolate | Standard amino acids | | | | |
|---|---|---|---|---|---|---|
| | | Alanine | Glutamic acid | Lysine | Glycine | Diaminopimelic acid |
| | | Rate of flow (Rf) | | | | |
| | | 0.74-0.80 | 0.66-0.73 | 0.63-0.67 | 0.55-0.60 | 0.36-0.41 |
| 1 | *K. palustris* TH28D | 0.80 | 0.73 | 0.66 | 0.60 | - |
| 2 | *K. palustris* H9 | 0.73 | 0.67 | 0.63 | 0.57 | - |
| 3 | *K. palustris* H21 | 0.74 | 0.67 | 0.63 | 0.58 | - |
| 4 | *K. rhizophila* TH121 | 0.78 | 0.72 | 0.67 | 0.61 | - |
| 5 | *K. rhizophila* TH122 | 0.78 | 0.72 | 0.67 | 0.61 | - |
| 6 | *K. rhizophila* TH211D | 0.79 | 0.72 | 0.67 | 0.60 | - |
| 7 | *K. rhizophila* TH216A | 0.79 | 0.73 | 0.67 | 0.61 | - |
| 8 | *K. rhizophila* TH218W | 0.79 | 0.71 | 0.67 | 0.61 | - |
| 9 | *K. rhizophila* TH219A | 0.79 | 0.72 | 0.67 | 0.61 | - |
| 10 | *K. rhizophila* TH226D | 0.78 | 0.72 | 0.67 | 0.61 | - |
| 11 | *K. rhizophila* TH227B | 0.79 | 0.73 | 0.67 | 0.61 | - |
| 12 | *K. rhizophila* TH236A | 0.79 | 0.73 | 0.68 | 0.61 | - |
| 13 | *K. rhizophila* H1 | 0.74 | 0.67 | 0.63 | 0.57 | - |
| 14 | *K. rhizophila* H3 | 0.73 | 0.66 | 0.63 | 0.57 | - |
| 15 | *K. rhizophila* R1 | 0.74 | 0.66 | 0.61 | 0.54 | - |
| 16 | *K. rhizophila* R3 | 0.74 | 0.67 | 0.62 | 0.55 | - |
| 17 | *K. rhizophila* R17 | 0.73 | 0.65 | 0.60 | 0.54 | - |

## Conclusions

The phenetic and phylogenetic analysis and clustering reflect food relatedness among strains of *K. palustris* and *K. rhizophila*, whereas whole cell protein bands profile showed low similarity among strains in this study.

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