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Isolation and characterization of an unknown bacterium containing plasmid-borne genes involved in carotenoid biosynthesis

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Isolation and Characterization of an Unknown bacterium containing plasmid-borne genes involved in carotenoid biosynthesis

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ABSTRACT (100-200 WORDS):

An unknown bacterium, designated AM-6, was isolated from soil near Montgomery Hall and characterized by analysis of its molecular, chemical, morphological and physiological properties. Research techniques from a wide array of biology and chemistry fields were utilized in order to obtain complete analysis of the bacterium. Phylogenetic examination of the 16S rRNA gene sequence showed that the bacterium was a member of the genus *Micrococcus*.

The strain's 16S rRNA gene sequence similarity with other species in the genus *Micrococcus* ranged from 97.94%-99.93%. The physiological properties of AM-6 differed from other *Micrococcus* species. Major cellular fatty acids of strain AM-6 were iso-$C_{15:0}$ (18%) and anteiso-$C_{15:0}$ (71.1%). AM-6 was found to contain MK-8(H_2) and MK-9(H_2) as the respiratory quinones. The bacterium contained a large plasmid; the exact size of it is yet to be determined. Elimination of the plasmid resulted in white colonies and 16S rRNA gene sequence analysis revealed that these white colonies are identical to the parent strain. The plasmid-encoded genes involved in carotenoid (pigment) biosynthesis, are yet to be identified. Based on the phenotypic, phylogenetic and chemotaxonomic data available AM-6 cannot be definitively labeled as a new species at this time. Further analysis is required to prove the novelty of this strain.

Abstract

An unknown bacterium, designated AM-6, was isolated from soil near Montgomery Hall and characterized by analysis of its molecular, chemical, morphological and physiological properties. Research techniques from a wide array of biology and chemistry fields were utilized in order to obtain complete analysis of the bacterium. Phylogenetic examination of the 16S rRNA gene sequence showed that the bacterium was a member of the genus *Micrococcus*. The strain's 16S rRNA gene sequence similarity with other species in the genus *Micrococcus* ranged from 97.94%-99.93%. The physiological properties of AM-6 differed from other *Micrococcus* species. Major cellular fatty acids of strain AM-6 were iso-$C_{15:0}$ (18%) and anteiso-$C_{15:0}$ (71.1%). AM-6 was found to contain MK-8(H_2) and MK-9(H_2) as the respiratory quinones. The bacterium contained a large plasmid; the exact size of it is yet to be determined. Elimination of the plasmid resulted in white colonies and 16S rRNA gene sequence analysis revealed that these white colonies are identical to the parent strain. The plasmid-encoded genes involved in carotenoid (pigment) biosynthesis, are yet to be identified. Based on the phenotypic, phylogenetic and chemotaxonomic data available AM-6 cannot be definitively labeled as a new species at this time. Further analysis is required to prove the novelty of this strain.

Background

Micrococcus is a genus of the family *Micrococcaceae*. Cells are usually catalase- and oxidase- positive and cocci arranged in tetrads. According to Dib *et al. (2013)*, the genus *Micrococcus* consists of ten species: *M. luteus, M. yunnanensis, M. lylae, M. antarcticus, M. cohnii, M. niistensis, M. lactis, M. terreus, M. flavus, and M. endophyticus. Micrococcus* species inhabit a wide variety of environments

including human skin, soil, glaciers, cheese, plants, dust, activated sludge, and sponges. Species of this genus are not known for being pathogenic but they are considered opportunistic pathogens because they usually infect organisms that have compromised immune systems. Certain *Micrococcus* species can contain extrachromosomal elements, which allow them to survive in harsh conditions. Large plasmids isolated from the genus *Micrococcus* have been shown to encode antibiotic resistance, tolerance of heavy metals, and degradation of cholesterol and hydrocarbons (Dib, *et al.* 2013).

Materials and Methods

Morphological properties

AM-6 was isolated from soil near Montgomery Hall. Routine cultivation of the strain was performed in Luria-Bertani (LB) medium at 37 °C for 48 hours. Colony morphology was determined by growing AM-6 on LB agar medium at 37 °C for 72 hours. Morphological properties of the cells were observed by light microscopy (x1000 magnification) and transmission electron microscopy (40,000x & 50,000x magnification).

Physiological characterization

AM-6 was subjected to various physiological tests in order to further support its phenotypic relationship with closely related bacterial species. Catalase activity was tested using 3% H_2O_2 (Leboff, *et al.*, 2010) and tested for oxidase activity using p-aminodimethyl aniline. Further physiological analysis was performed using BiOLOG GENIII MicroPlate™ identification systems and MicroLog 3 v. 5.1.1 software. Procedure was carried out as explained in the BiOLOG GEN III MicroPlate™ manufacturer's instruction

manual. Incubation of the plates containing AM-6 wild type and *Micrococcus luteus* occurred at 35 °C for 32 hrs. Results were examined using BiOLOG Microstation after 12, 24, and 32 hrs. Numerous trials using the BiOLOG system confirmed the results.

Determination of optimum growth conditions

Growth temperature was observed by inoculating 50 µl of overnight culture (centrifuged and suspended in 1mL of LB medium) in LB broth medium (30 mL) at various temperatures (20, 23, 28, 30, 35, 37, 39, 42, 43) for 72 hours. Growth was checked every 24 hrs using a Klett-Summerson Photoelectric Colorimeter. Investigation of optimal growth pH was carried out by inoculating the strain into 30 mL of LB medium at pHs ranging from 4.0-12.0 (at 0.5 pH unit increments). Cultures were grown for 48 hrs and the turbidity was checked every 24 hrs using the previously described colorimeter. Salt tolerance was analyzed in LB media supplemented with 1.0-15% (at 1% increments) NaCl for 48 hrs (Zhang et. al, 2010).

Antibiotic Susceptibility

The Kirby-Bauer method (Leboff *et al.*, 2010) was utilized to determine the antibiotic susceptibility of AM-6. Overnight cultures were centrifuged, 50 µl of cells were spread ~~plated onto LB agar medium~~, and antibiotic discs were added. The following antibiotics were tested: cephalothin (30ug), methicillin (75 ug), mezlocillin (75ug), cefazolin (30 ug), cefonicid (30 ug), amikacin (30ug), piperacillin (100 ug), gentamycin (10 ug), tobramycin (10 ug), nalidixic acid (30 ug), kanamycin (30 ug), vancomycin (30 ug), tetracycline (30 ug), erythromycin (15ug), novobiocin (30ug), rifampin (5 ug), streptomycin (10 ug), penicillin (10 IU/E/UI), polymyxin B (300 units), bacitracin (10 units), chloramphenicol (30 ug).

Plasmid Isolation/Curing

AM-6 was inoculated in 5ml of LB medium and grown for 48hrs at 37°C. The culture was centrifuged, resuspended in a buffer solution of 50mM glucose/10mM EDTA/10mM Tris-HCL (pH 8.0) and 20 µl of lysozyme was added (100 mg/ml). Lysis solution of 0.2M NaOH/1% sodium dodecyl sulfate was added to the resuspension and incubated at room temperature for 5 minutes. Next, 150 µl of 7.5 M ammonium acetate was added and immediately followed by 150uL of chloroform. The solution was mixed by inversion, chilled on ice for 10 minutes, and then centrifuged for 10 minutes. The supernatant was transferred to 200 µl of precipitation solution (30% polyethylene glycol 8000/1.5 M NaCl), mixed by inversion and then chilled on ice for 15 minutes. This mixture was centrifuged and the supernatant was decanted. The pellet was resuspended in 20 uL of water and dissolved overnight at 4 °C (Heringa, *et al.*, 2007).

Plasmid curing was performed by growing the wild type strain at 42 °C. AM-6 was grown in 50mL of LB medium at 42 °C for 48 hrs. The two-day growth was transferred and grown in fresh medium. This process was repeated five times and after the fifth round, a serial dilution was performed and plated on LB agar medium. (Dib *et al.*, 2010).

Quinone Analysis

The strain was grown in 4 L of LB broth at 37 °C for 48 hours. The culture was pelleted and freeze-dried using VirTis BenchTop 4K Freeze Dryer. Next, 160 mL of chloroform-methanol (2:1) was added to 800 mg of dried cells in a 250 mL Erlenmeyer flask. The mixture was placed in complete darkness and stirred overnight using a magnetic stirring bar. The cell suspension was filtered through Whatman No. 1 filter paper. Drying of the filtrate was performed by rotary evaporation and acetone was utilized to

resuspend the residue and prepare the sample for HPLC (Meganthan & Coffell, 1985). High performance liquid chromatography (HPLC) and mass spectrometry (MS) were used to identify the quinone. Mass to charge ratios of known quinones were compared with mass spectrometry data obtained from the isolate.

Phylogenetic Analysis

The 16S rRNA gene was amplified and sequenced from AM-6 wild type and cured strain. Genomic DNA was isolated from both strains using the Promega Wizard® Genomic DNA Purification Kit. Isolated genomic DNA was used as a template to amplify the 16S rRNA gene by polymerase chain reaction (PCR). Conserved standard primers 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1522R (5'AAGGAGGTGATCCAGCCGCA) were used. The PCR was performed using 25 µl HotStart IT FideliTaq Polymerase, 5 µl reverse and forward primer, 15 µl H_2O, and 2 µl of genomic DNA. Template DNA was amplified using a Gene Amp® PCR system 2700 with the following program: 94°C for 3 min, 40 cycles of 94°C for 30 s, 45°C for 60 s, and 72°C for 3.5 min, and a final extension cycle at 72°C for 5 min (Winker, 1991). Promega Wizard® Plus SV Minipreps was used for purification of PCR product. Sequencing was performed by ACGT, Inc.

The strain's 16S rRNA gene similarity was calculated using EzTaxon-e. (Kim, et al. 2012). Sequence similarity was subjected to multiple alignment using Clustal X version 2.0 alignment software (Thompson, *et al.* 1997). The construction of a phylogenetic tree was performed using MEGA v. 5.10. The parameters used for tree construction were neighbor-joining, pairwise deletion to fix gaps, and Tamura-Nei model for 1,000 boostrap replicates (Saitou & Nei, 1987).

Results

Morphology

The colonies of AM-6 wild type were yellow-pigmented, round, convex, and smooth. The cured AM-6 strain formed colonies that were white, round, convex and smooth. Analysis of cell morphology led to the discovery that they were cocci arranged in tetrads. Electron and light microscopy analysis confirmed the tetrad arrangement.

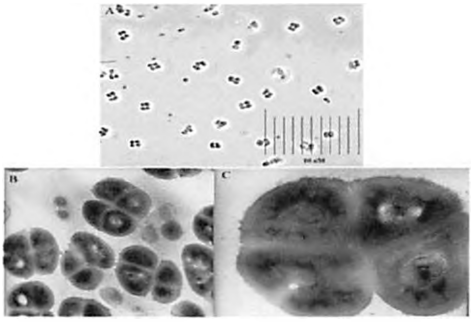

Figure 1: Top: Phase contrast micrograph of bacterium grown on LB broth at 30°C for 24 h **Bottom.** Transmission electron micrograph of a field of tetrads; B. Magnification 40,000X; C, single tetrad magnification: 50,000x.

Phenotypic characterizations

The bacterium was catalase positive, oxidase negative, non-motile, and non-spore forming. Biolog analysis showed that AM-6 was capable of metabolizing dextrin, D-turanose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, and glycerol. Sugars with borderline results were α-D-Glucose and 3-methyl Glucose. It was discovered that AM-6 was able to metabolize various amino acids: D-Aspartic acid, L-Arginine, L-Aspartic acid, L-Glutamic acid, L-Histidine, L-Pyroglutamic acid, and L-Serine. As seen from the Table 1 & 2, there are major

differences between the abilities of AM-6 and *Micrococcus luteus* to metabolize various sugars, amino acids, carboxylic acids, fatty acids, and esters.

Table 1 &2: Biolog Gen III Microplate™ carbon source metabolism results obtained for AM-6 wild type and *M. luteus*.

Carbon Source	AM-6	*M. Luteus*
Glycyl-L-Proline	+/-	-
L-Alanine	-	+
L-Arginine	+	+/-
L-Aspartic Acid	+	-
L-Glutamic Acid	+	+
L-Histidine	+	-
L-Pyroglutamic acid	+	+
L-Serine	+	-
Pectin	+	+
D-Galactouronic acid	+	+
L-Galactonic Acid Lactone	-	-
D-Gluconic Acid	+	+
D-Glucuronic Acid	+	+
Glucuronamide	+	+
Mucic Acid	+/-	+/-
Quinic Acid	+	-
D-Saccharic Acid	+/-	-
p-Hydroxy-Phenylacetic	+/-	+
Methyl Pyruvate	-	-
D-Lactic Acid Methyl Ester	+	-
L-Lactic Acid	+	+
Citric Acid	+	-
α-Keto-Glutaric Acid	-	+/-
D-Malic Acid	+	-
L-Malic Acid	+	+/-
Bromo-Succinic Acid	-	-
Tween 40	+	+/-
γ-Amino-Butryric Acid	+	-
α-Hydroxy-Butyric Acid	+	-
α-Hydroxy-D-L-Butyric Acid	+	-
α-Keto-Butyric Acid	+	+
Acetoacetic acid	+	+
Propionic Acid	+	-
Acetic Acid	+	+/-
Formic Acid	-	-
+ = Positive, +/- = Borderline, - = Negative		

Carbon Source	AM-6	*M. Luteus*
Dextrin	+	+
D-Maltose	-	+
D-Trehalose	-	+
D-Cellobiose	-	-
Gentiobiose	-	-
Sucrose	-	+
D-Turanose	+	+
Stachyose	-	-
D-Raffinose	-	-
α-D-Lactose	-	-
D-Melibiose	-	+
β-Methyl-D-Glucoside	-	-
D-Salicin	-	-
N-Acetyl-D-Glucosamine	-	-
N-Acetyl-β-D-Mannosamine	-	-
N-Acetyl-D-Galactosamine	-	-
N-Acetyl Neuraminic acid	-	-
α-D-Glucose	+/-	+
D-Mannose	+	+
D-Fructose	+	-
D-Galactose	+	+
3-Methyl Glucose	+/-	+
D-Fucose	+	+
L-Fucose	+	+
L-Rhamnose	+	+
Inosine	-	-
D-Sorbitol	-	-
D-Mannitol	-	-
D-Arabitol	-	-
myo-Inositol	-	-
Glycerol	+	+
D-Glucose-6-PO4	+	+/-
D-Fructose-6-PO4	+	+
D-Aspartic Acid	+	-
D-Serine	-	-
Gelatin	+	-
+ = Positive, +/- = Borderline, - = Negative		

Determination of optimum growth conditions

The strain grew at 23-42 ^{0}C, with an optimal temperature of 37 ^{o}C. Also, it was discovered that the pH range for growth was pH 6-10 and the optimum pH was 8.5-9.0. Growth occurred in LB media with no NaCl present and the bacterium could tolerate salt concentrations up to 13.5%.

Antibiotic Susceptibility

Antibiotic susceptibility testing led to the discovery that AM-6 was resistant to methicillin (5ug), tobramycin (10ug), and nalidixic acid (30ug).

Table 3: Antibiotic susceptibility of AM-6 wild type strain.

Antibiotic	AM-6
Cephalothin (30ug)	S
Methicillin (5ug)	R
Mezlocillin (75ug)	S
Cefazolin (30ug)	S
Cefonicid (30ug)	S
Amikacin (30ug)	S
Piperacillin (100ug)	S
Gentamycin (10ug)	S
Tobramycin (10ug)	R
Nalidixic Acid (30ug)	R
Kanamycin (30ug)	S
Vancomycin (30ug)	S
Tetracyclin (5ug)	S
Erythromycin (15ug)	S
Novobiocin (5ug)	S
Rifampin (5ug)	S
Streptomycin (10ug)	S
Penicillin (10IU/IE/UI)	S
Polymyxin B (300ug)	S
Bacitracin (10units)	S
Chloramphenicol (30ug)	S
R = Resistant, S = Susceptible	

Phylogenetic Analysis

Phylogenetic analysis of 16S rRNA gene sequence clearly revealed that AM-6 is a member of the genus *Micrococcus*. Sequence similarity calculation showed that AM-6 was most closely related to *Micrococcus yunnanensis* YIM 65004T (99.93%), *Micrococcus luteus* NCTC 2665T (99.71%), and *Micrococcus endophyticus* YIM 56238T (99.36%), *Micrococcus antarcticus* T2T (98.43%), *Micrococcus lylae* DSM 20315T (98.29%), *Micrococcus flavus* LW4T (98.19%), and *Micrococcus cohnii* WS4601T (97.94%). Neighbor-joining tree revealed that AM-6 formed a distinct branch with the genus *Micrococcus* (96%). AM-6 branched most frequently with *M. luteus* and *M. lylae*; however, boostrap values were not high enough to assign AM-6 its own distinct branch.

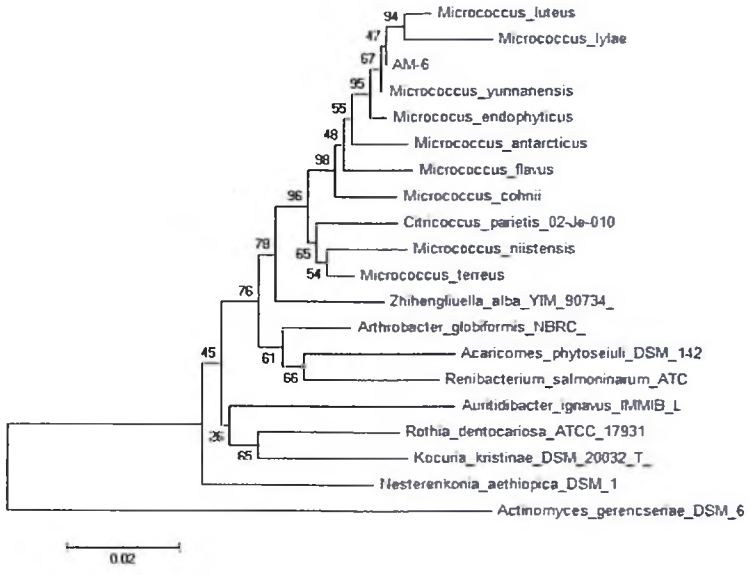

Figure 2: Neighbor joining tree based on 16S gene sequence similarity between AM-6 and closely related taxa. Bootstrap values are based on 1000 replicates using pairwise deletion to eliminate gaps and the Tamura-Nei model. Only bootstrap values greater than 50% should be used.

Chemotaxonomic Analysis

Cellular fatty acid analysis revealed that AM-6 had very similar components to other *Micrococcus* species. All species in this genus, including AM-6, contained iso-$C_{15:0}$ and anteiso-$C_{15:0}$ as their major

cellular fatty acids. Slight differences occurred in the minor cellular fatty acid compositions of the various *Micrococcus* species. The data in Table 4 shows that the minor fatty acids were very similar to *Micrococcus luteus* DSM 20030^T. The presence of anteiso-$C_{17:0}$ in AM-6 is not seen in the other species of this genus.

Table 4: Cellular fatty acid composition of various *Micrococcus* species. Taxa: 1, strain AM-6; 2, *M.yunnanensis* DSM 21948T (Zhao, et al., 2010); 3, *M. luteus* DSM 20030T (Wieser, *et al.*, 2002); 4, *M. endophyticus* DSM 17945T (Chen, et al., 2009); 5, *M. antarcticus* AS 1.2372T (Liu, *et al.*, 2000); 6, *M. lylae* DSM 20315T (Wieser, *et al.*, 2002); 7, *M. cohnii* DSM 23974 (Rieser, *et al.*, 2013); 8, *M. terreus* (Zhang, et al. 2010); 9, *M. flavus* JCM 14000T (Liu, *et al.*, 2007)

Taxa	i-$C_{14:0}$	$C_{14:0}$	i-$C_{15:0}$	i-$C_{15:1}$	ai-$C_{15:0}$	ai-$C_{15:1}$	i-$C_{16:0}$	$C_{16:0}$	ai-$C_{17:0}$	ai-$C_{17:1}$
1	-	1.9	18	-	71.1	-	2.4	-	2.3	-
2	-	-	13.04	-	61.98	-	14.25	-	-	-
3	-	3.9	29.1	-	49.7	-	2.1	2.1	3.1	-
4	2.59	-	30.95	-	53.75	-	1.42	-	-	1.36
5	-	-	19.9	-	49.7	12.3	-	-	-	-
6	4.7	1.2	33.33	-	52.2	-	4.2	-		1
7	4.3	-	10.5	-	78.2	-	4.3	-	-	-
8	3.06	-	13.1	-	78.2	-	3.55	-	-	-
9	1.74	1.39	31.65	19.21	32.15	-	-	2.05	-	-
i = iso ai= anteiso										

The quinone composition was determined using HPLC and MS (LC-MS). Using known menaquinone mass-to-charge ratios (m/z), AM-6 was found to contain MK-8 (H_2) and MK-9 (H_2) as respiratory quinones. Table 5 shows the comparative analysis with known major quinone compositions of various *Micrococcus* species. MK-8 (H_2) is present in every species of this genus, however; MK-9 (H_2) is not present as a major quinone in any other species. The presence of MK-9 (H_2) as a major quinone in AM-6 must be tested further to prove these results.

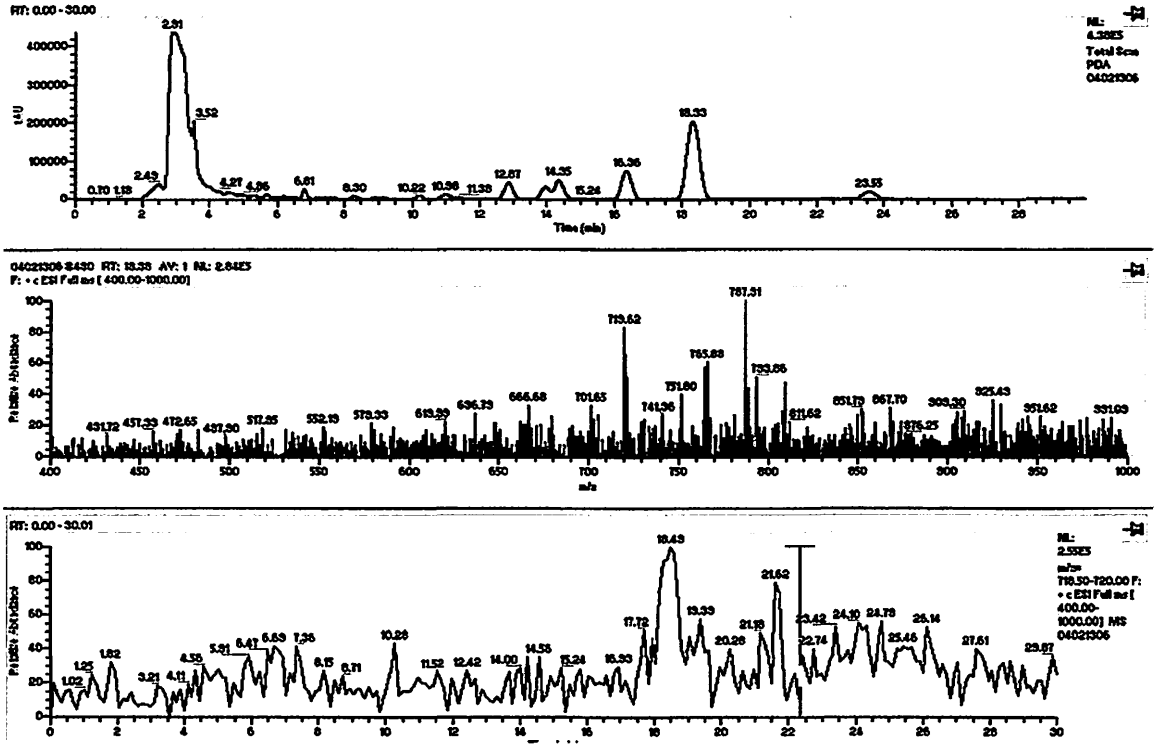

Figure 3: Top: High performance liquid chromatography (HPLC) of AM-6 quinone sample.

Middle & Bottom: Mass spectrometry of quinone sample. Peaks were compared with known menaquinone molecular weights. Peaks at 719.62 and 787.31 m/z matched known values of MK-8 (H2) and MK-9 (H2) respectively.

Table 5: Known respiratory quinones for various *Micrococcus* species. Taxa: 1, strain AM-6; 2, *M.yunnanensis* DSM 21948T (Zhao, et al., 2010); 3, *M. luteus* DSM 20030T (Wieser, *et al.*, 2002); 4, *M. endophyticus* DSM 17945T (Chen, et al., 2009); 5, *M. antarcticus* AS 1.2372T (Liu, *et al.*, 2000); 6, *M. lylae* DSM 20315T (Wieser, *et al.*, 2002); 7, *M. cohnii* DSM 23974 (Rieser, *et al.*, 2013); 8, *M. terreus* (Zhang, et al. 2010); 9, *M. flavus* JCM 14000T (Liu, *et al.*, 2007)

Taxa	MK-7	MK-8	MK-7 (H_2)	MK-8 (H_2)	MK-9 (H_2)
1	-	-	-	+	+
2	-	-	+	+	-
3	-	+		+	
4	-	-	+	+	-
5	-	+	-	+	-
6	-	-	-	+	-
7	-	-	+	+	-
8	-	-	+	+	-
9	+	+	+	+	-

+ = Major quinone, - = Minor or no quinone

Plasmid

Due to previous findings of large plasmids within the *Micrococcus* genus, AM-6 was tested for the presence of a plasmid. The isolation procedure resulted in the extraction of what is believed to be a large plasmid. After curing procedure was performed, white colonies were discovered. The morphology of these colonies were examined by light microscopy and it was discovered that the cells were cocci and formed tetrads. Comparison of the 16S rRNA gene sequence for the cured strain (white) with the wild type strain (yellow) revealed that they were identical. These plasmid results are preliminary and thus more testing must be done for confirmation.

Conclusion

An unknown bacterium was isolated from soil near Montgomery Hall. 16S rRNA gene analysis showed sequence similarity of 97.94%-99.93% with various *Micrococcus* species. The cells were gram-positive, non-endospore forming, non-motile, aerobic, and cocci arranged in tetrads. Colonies were yellow, round, convex, and smooth. The strain grew at a temperature range of 24-42 °C with optima at 37 °C. Also, optimal growth occurred at a pH of 9.0 with a range of 6-10 and AM-6 was able to tolerate 13.0% NaCl concentration. Catalase and oxidase tests were positive and negative respectively. Gelatin was hydrolysed and AM-6 was able to use dextrin, D-turanose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, and glycerol as carbon sources. The bacterium was able to metabolize various amino acids: D-Aspartic acid, L-Arginine, L-Aspartic acid, L-Glutamic acid, L-Histidine, L-Pyroglutamic acid, and L-Serine. The strain carries MK-8 (H_2) and MK-9 (H_2) as respiratory quinones and the major cellular fatty acid composition is iso-$C_{15:0}$ (18%) and anteiso-$C_{15:0}$ (71.1%).

It was discovered that the bacterium contained a large plasmid. Curing of the plasmid resulted in a white colony which, was confirmed to be identical to the AM-6 wild type sequence by 16S rRNA gene analysis. These preliminary findings have led to the hypothesis that the strain's yellow pigmentation is due to plasmid-borne genes. Various genes involved in the biosynthesis of the bacterium's pigment may be encoded on the plasmid. Since it is possible that a spontaneous mutation could have occured in the cell's genomic DNA, further analysis of the extrachromosomal elements in the strain must be performed to rule out this possibility.

Based on phenotypic and phylogenetic analysis it can be concluded that AM-6 is a member of the genus *Micrococcus*. The strain showed various phenotypic, morphological, and possibly chemotaxonomic differences when compared to *Micrococcus luteus* and previous data from other closely related *Micrococcus* species; however, at this time, there is not enough data to definitely conclude that AM-6 is a new species. Further phenotypic testing with other known *Micrococcus* species, chemotaxonomic analysis, and DNA-DNA hybridization with closely related species are required in order to confirm the novelty of AM-6.

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