Identification and characterization of *Rhodopseudomonas* spp., a purple, non-sulfur bacterium from microbial mats

Sharifeh Mehrabi a,*, Udoudo M. Ekanemesang a, Felix O. Aikhionbare b, K. Sean Kimbro a, Judith Bender a

a Department of Biological Sciences, Clark Atlanta University, Atlanta, GA 30314, USA

b Department of Microbiology/Biochemistry/Immunology, Morehouse School of Medicine, Atlanta, GA 30310, USA

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Abstract

A species of facultative photo-organotrophic, purple, non-sulfur bacterium was isolated from mixed-species microbial mats, characterized and examined for metal tolerance and bioremediation potential. Contributing mats were natural consortia of microbes, dominated by cyanobacteria and containing several species of bacteria arranged in a laminar structure, stabilized within a gel matrix. Constructed microbial mats were used for bioremediation of heavy metals and organic chemical pollutants. Purple, non-sulfur bacteria are characteristically found in lower strata of intact mats, but their contributing function in mats survival and function by mediating the chemical environment has not been explored. The gram-negative rod-shaped bacterium, reported here, produced a dark red culture under phototrophic conditions, reproduced by budding and formed a lamellar intracytoplasmic membrane (ICM) system parallel to cytoplasmic membrane, which contained bacteriochlorophyll a and carotenoids. This strain was found to have multiple metal resistances and to be effective in the reductive removal of Cr(VI) and the degradation of 2,4,6-trichlorophenol. Based on the results obtained from morphology, nutrient requirements, major bacteriochlorophyll content, GC content, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) profile and 16S-rDNA phylogenetic analysis, this member of the microbial mats may be identified as a new strain of the genus *Rhodopseudomonas*. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Species of the genus *Rhodopseudomonas* constitute the majority of phototrophic purple non-sulfur bacteria, and are characterized as rod-shaped, motile cells that show polar growth and asymmetrical division or budding as a mode of reproduction [1]. They form lamellar intracytoplasmic membranes that are adjacent and parallel to cytoplasmic membrane and contain photosynthetic pigments under phototrophic conditions [2]. The colors of cell suspensions of many species grown under phototrophic conditions vary from species to species, indicating the presence of major carotenoid types (maximum absorption at 450–550 nm) and bacteriochlorophylls [3]. Absorption spectra of cell suspensions yield primary information on predominant bacteriochlorophylls and carotenoids. This color trait is useful in initial characterization of these species [2]. The major carotenoids are lycopene, rhodopin (463, 490, 524 nm), spirilloxanthin (486, 515, 552 nm), okenone (521 nm) and spheroidene (450, 482, 514 nm). The spirilloxanthin series (lycopene, rhodopin, spirilloxanthin) as the major component gives a pink or red color, increasing amounts of rhodopin turn the color to red–brown, okenone (521 nm) results in purple–red. The spheroidenone series gives a green or brownish red and greenish brown under reducing conditions [2,3].

*Rhodopseudomonas* species have shown a high degree of morphological and structural similarities, however, they are phylogenetically quite diverse. Comparisons of rRNA sequences, DNA–DNA and DNA–RNA hy-
broidization have demonstrated that these bacteria belong to several different lines of descent within the alpha-2 subclass of Proteobacteria [3]. Morphological, cytological and physiological properties of the genus *Rhodopseudomonas* have been summarized by Imhoff et al. [4]. Recent phylogenetic studies of *Rhodopseudomonas* species demonstrated that *Rhodopseudomonas palustris* is more closely related to some chemotrophic taxa, such as *Afipia felis*, *Bradyrhizobium japonicum*, *Blastobacter denitrificans* and *Nitrobacter winogradskyi*, than to any of the other *Rhodopseudomonas* species for which rDNA sequences are available [5,6].

Phototrophic non-sulfur bacteria have been used in sewage treatment processes [7,8], for biomass and vitamin production [9] and for production of molecular hydrogen [10]. In bioenergetic research, they have been used as a cell-free system for performing photosynthesis [11,12]. Biodegradation of aromatic compounds by *Rhodopseudomonas* species such as benzoate [13], dihydroxylates, methoxyolate [14] and 4-hydrobenzoate [15] have been reported. Non-sulfur bacteria display a high resistance to heavy metals [16], giving these cells another potential application in bioreclamation of rare-earth metals.

In attempts to isolate the bacterial members of mats and elucidate their roles in bioremediation, we isolated and partially characterized a phototrophic, purple, non-sulfur bacterium reported in this study. The mats were developed for bioremediation and composed of *Oscillatoria* spp. The contribution of this strain in microbial mats bioremediation properties will be reported in future.

2. Materials and methods

2.1. Bacterium source and isolation technique

The bacterium was isolated from a mature microbial mat used for metal sequestration and organic pollutant degradation experiments. The development and application of microbial mats have been reported previously [17,18,20–22]. Samples of microbial mat were blended in sterile saline solution (NaCl 8.5 g/l) and serially diluted. Colony count pour plates were prepared from each dilution in nutrient agar medium (Dickerson Microbiology Systems, Cokeysville, MD), incubated at room temperature in the dark and anaerobically under 60 W incandescent light in an anaerobic chamber (Forma Scientific, Marietta, OH). Several red or red–brown colonies of different sizes and textures appeared after five to seven days incubation under anaerobic/light conditions. The most dominant red colony was selected for purification. Several representatives of single red colonies were individually transferred into screw cap tubes filled with nutrient broth medium and incubated under the light at room temperature for enrichment. To ensure the purity of cell culture, the streak dilution plates were prepared from each tube containing the originally isolated colony on nutrient agar medium, and checked for purity.

2.2. Determination of nutritional and physiological characteristics

The stock solutions of different carbon sources were filter-sterilized and added to the AT salt medium [2] to a final concentration of 1–2 g/l. The pH of the media was adjusted to 7 with HCl or NaOH for routine analysis or to 5, 6, 7 or 8 for pH profile analysis. Heterotrophic cultures were grown in 100 ml of medium in 250 ml flasks incubated in a rotary shaker at 150 rpm in the dark. Phototrophic cultures were grown in completely filled 250 ml screw cap culture bottles and incubated under 60 W incandescent bulbs at a distance of 60 cm in an anaerobic chamber. The growth was monitored turbidimetrically, using a Beckman DU 650 UV/VIS scanning spectrophotometer (Beckman, Fullerton, CA) at 600 nm. The number of colony forming units (CFU) was determined by the spread plate count method using serial dilutions. The growth rate constant (K), the number of generations (n) and the mean generation time (g) were calculated using standard mathematical growth equations [24]. The measurement of optimum growth temperature was carried out in nutrient broth medium, in a water bath set to the desired temperature. Assay for formation of intracellular sulfur globules was performed according to the method described by Balows et al. [2]. Briefly, the cells were incubated on a microscopic slide for 20 min with neutralized Na,S solution and monitored for intracellular sulfur globules formation. *Chromatium vinosum* (ATCC 17899), a sulfur purple bacterium was used as a positive control. All experiments were performed in triplicate.
2.3. Absorption spectra and electron microscopy

Determination of the predominant bacteriochlorophyll and the carotenoid composition of the isolate were performed as described by Balows et al. [2]. The absorption spectra of cell suspensions in 4 M sucrose were recorded against a blank of 4 M sucrose solution using a Beckman DU 650 UV/VIS scanning spectrophotometer. The approximate maximum absorption of major peaks was determined. A transmission electron microscope (TEM, Hitachi H-6000), set at 0.3 nm point-to-point resolution and 50 000 × magnification, was used to examine the isolate. Standard microscopic methods were used for slide preparation and Gram staining.

2.4. Reductive removal of Cr(VI)

Cells were grown to the late log phase in nutrient broth harvested, washed and resuspended in 100 ml of minimal salt medium at cell density equivalent to optical density reading of 1.5 at 600 nm. Varying concentrations of dichromate solution were added to the cells. Aliquots were removed at time intervals, filtered and analyzed for hexavalent chromium. The concentration of Cr(VI) was determined colorimetrically by reaction with diphenylcarbazide in acid solution as described in section 3500 of the Standard Method for Examination of Water and Wastewater (American Public Health Association, 1995). An aliquot of 2 ml of sample was added to 9.8 ml of 0.2 N H2SO4, followed by the addition of 0.5 ml of diphenylcarbazide reagent (0.5% in acetone, Labchem Inc., Pittsburgh, PA). The absorbance of the solution was measured at 540 nm using a Beckman DU 650 UV/VIS scanning spectrophotometer.

2.5. Degradation of trichlorophenols

Cells were grown in nutrient broth to the late log phase, harvested, washed and resuspended in 100 ml of minimal salt medium at cell density equivalent to optical density reading of 1.5 at 600 nm. 2,4,6-Trichlorophenol (100 µg/ml) was added to the culture flasks and incubated in an anaerobic chamber at 30 °C on a rotary shaker at 150 rpm. At time intervals aliquots of the culture (2.0 ml) were removed; cells were removed by centrifugation. Equal volume of methanol was added to the supernatant and filtered through a 0.45 µm pore-size filter (Millipor, Bedford, MA) into a HPLC sample vial. Samples were analyzed using a Beckman System Gold HPLC equipped with a model 502 autosampler, 126 solvent module, diode array detector and c18 column (15 cm × 4.6 mm; particle size 5 µm). Injection volume was 20 µl. The mobile phase was methanol/water (60:40) at a flow rate of 1 ml/min. Peaks were quantified by external standards using system Gold.

2.6. Sample source and GC content analysis

Seven species of Rhodopseudomonas that share phenotypic characteristics with the isolate were purchased from ATCC (American Type Culture Collection) (Table 1). These strains were cultured in media recommended by ATCC under phototrophic conditions. Cells were harvested at their exponential growth phase. The G + C base content of the isolate was determined by the thermal denaturarion method as described by Marmur and Doty [25].

2.7. DNA preparation and 16S-rDNA sequence analysis

DNA extraction was performed using the methods described by Maniatis et al. [26]. DNA was then precipitated with chilled ethanol, washed with 70% ethanol, dried and finally dissolved in TE (10:1) and stored at −20 °C until diluted to about 100 ng/µl prior to use.

Amplification of 16S ribosomal DNA (rDNA) was performed on the isolated DNA using primers (5′-CAGCCAGCCCGTGAATAC-3′) and (5′-CCGT-CAATTCTTTGAGTT-3′). The PCR reaction mix was performed as previously described [27,28]. Amplified products were resolved on 1.5% agarose gel, then excised from the gel and purified. The purified products were cloned into pGEM-T (Promega) according to manufacturer’s instructions and subsequently sequenced using Big Dye Terminator chemistry (ABI, Foster, CA). The sequences were assembled using Autoassembler and sequence navigator (ABI, Foster, CA). A 407 bp 16S-rDNA sequence was obtained from this study.

2.8. RAPD-PCR analysis

A total of 20 random and previous 16S-rRNA sequences of Rhodopseudomonas species were used in the selection of RAPD primers, then obtained from Genosys Biotechnologies Inc. (Woodlands, TX). All

<table>
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<th>Sample</th>
<th>Species</th>
<th>ATCC</th>
<th>Reference</th>
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<tr>
<td>1</td>
<td>Rps. palustris (RPS p)</td>
<td>17001</td>
<td>[31]</td>
</tr>
<tr>
<td>2</td>
<td>Rps. acidiphila (RPS a)</td>
<td>25092</td>
<td>[31]</td>
</tr>
<tr>
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<td>Rps. rutila (RPS r)</td>
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<td>[33]</td>
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<td>Rps. virida (RPS v)</td>
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<td>[31]</td>
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<td>6</td>
<td>Rps. marina (RPS m)</td>
<td>35675</td>
<td>[34]</td>
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<tr>
<td>7</td>
<td>Isolate</td>
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RAPD primers used in this study were 10-mers, with a 60% or 70% G+C content. RAPD-PCR reactions were obtained in 25 μl volume similar to previously described [29]. Amplified PCR products and controls were resolved in 1.5% agarose gels. All the resultant bands were scored in reference to the 100 bp molecular weight ladder (GibcoBRL, Gaitherburg, MD) as either present or absent across the lanes.

2.9. Phylogenetic analysis

407 bp of the 16S-rDNA sequence of the isolate was matched to other Rhodopseudomonas spp. obtained from GenBank databases. Names and accession numbers are as follows: Rhodopseudomonas spp. B29, AB027692; Uncultured alpha proteobacterium A, AB035489; Uncultured alpha proteobacterium, AB035490; Bradyrhizobium spp. Mml-3, AF159438; Bradyrhizobium spp. Pel-3, AF159436; Bradyrhizobium spp. Pe4, AF159437; Bradyrhizobium spp. DesB1, AF178436; Bradyrhizobium spp. jwc91-2, AF178437; Bradyrhizobium spp. th-b2, AF178438; Bradyrhizobium spp. ORS2012, AF230721; Bradyrhizobium spp. ORS285, AF230722; Bradyrhizobium elkanii strain SEMIA 5019, AF237422; Bradyrhizobium spp. STM461, AF239254; Bradyrhizobium spp. ORS278, AF239255; Bradyrhizobium elkanii PRY52, AF239846; Bradyrhizobium japonicum, AF293382; Bradyrhizobium spp. strain Cj3-3, AF321215; Bradyrhizobium liaoningense strain 2281, AF363132; Bradyrhizobium japonicum strain USDA 123, AF363151; Apilia genosp. U87771; Rhizospere soil bacterium isolate RSI-33, AJ252600; Bradyrhizobium spp. MSDJ5726, AF363149; Bradyrhizobium elkanii strain USDA 94, AF363152; Bradyrhizobium genosp. J. Z94820; Rhodopseudomonas palustris, D89811; Rhodopseudomonas spp. g-c 16S AF123086; R. palustris, X87279. The program Clustal W [30] was used to create a multiple-sequence alignment of the sequences and to construct the tree from the alignment. Bootstrapped distance matrix analysis was performed using Clustal W with 1000 bootstrapping trials. We used the PHYLIP program DRAWTREE to draw the tree from the output of the Clustal W program.

3. Results

3.1. Morphology and phototrophic properties

The isolate was a gram-negative rod-shaped motile bacterium with a length of 2.0–2.4 μm. The cells divided
asymmetrically by budding. Bud formation at the end of the mother cell gives a dumbbell-shaped appearance before separation of the daughter cell [1] (Fig. 1). The electron micrographs of thin sections of phototrophically grown cells showed a lamellar intracytoplasmic membrane (ICM) system (Fig. 2). The bacteria form smooth, round and dark red colonies with a diameter of 2–3 mm on agar media and red cell suspensions in liquid media under phototrophic conditions. The strain formed photosynthetic pigments when grown under anoxygenic phototrophic conditions. Absorption spectra of cells suspended in sucrose had three major peaks at 593, 805 and 871 nm, indicating the presence of bacteriochlorophyll a (590, 800–900 nm), and three peaks at 463, 490 and 530 nm, indicating the presence of lycopene and rhodopin. There were no absorption peaks for bacteriochlorophylls b (400, 605, 840 and 1025–1035 nm) and c (660–668 nm), which are characteristic of purple non-sulfur bacteria (Fig. 3) [2,3].

### 3.2. Physiological and metabolic properties

Optimal growth occurred at 30–35 °C and at pH 6–8 under both heterotrophic and phototrophic conditions. Phototrophic growth was observed on several carbon and electron donor sources (Table 2). The growth of the bacteria in nutrient broth was significantly higher than in minimal salt media with a single carbon source. The $K$ values (mean growth rate constants) of the bacterial growth in nutrient broth and other carbon substrates under phototrophic and heterotrophic conditions are presented in Table 3.

### 3.3. Strain identification

The isolate was sent to two commercial organizations, ATCC (Bethesda, MD) and Analytical Services Inc. (Essex Junction, VT) for their independent confirmation of our identification. Analytical Services was unable to obtain a match on its microbial identification.
system (MIS) database using the fatty acid profile. Based on microscopic examination, cultural requirements and metabolic properties, ATCC identified the bacterium as an unknown species of *Rhodopseudomonas* genus.

### 3.4. Bioremediation property

The bacterium reduced Cr(VI) to Cr(III). The reduction was rapid at lower concentrations of Cr(IV) and decreased at higher concentrations as toxicity of Cr(IV) increased. The reduction rates were as follows: 10 mg/l per 2 h and 80 mg/l per 2 days. TCP degradation rate was about 10 mg/l per day. The metabolic products of 2,4,6-TCP and 2,4-DCP, 4-CP also appeared in approximately 3 h and 2 days and disappeared in 4 and 8 days, respectively. These products were identified using known standards. 4,6-DCP, 2,6-DCP, 2-CP and 6-CP were not detected, suggesting that groups at positions 6 and 2 are most labile.

### 3.5. RAPD

While 20 RAPD primers produced multiple amplification products for the seven bacterial species (Fig. 4), three primers: SMO1 (5'-ACGGTGCCCTG-3'), SMO2 (5'-GAGATCCGCG-3') and SMO3 (5'-CGGGTCGATC-3') produced the most variable banding profile. RAPD-PCR produced distinguishable bands with the studied species (lane 7) from all other six species (lanes 1, 2, 3, 4, 5, 6).

### 3.6. Phylogeny

To classify the isolate, we used Clustal W [29] to align the 407 bp sequence obtained from the isolate with several non-sulfur alpha-2 Proteobacteria groups. From this alignment, we construct a rooted phylogenetic tree (Fig. 5). Phylogenetic analysis of this isolate using the 16S-rDNA sequence data suggests that the isolate was an unidentified species that is closely related to *Bradyrhizobium* spp. and *Rhodopseudomonas palustris*.

### 4. Discussion

One of the objectives of this study was to identify and characterize some of the dominant species in a synthetic microbial mat designed for bioremediation applications. Results from this investigation provided information on a heretofore-unidentified strain of *Rhodopseudomonas* spp., a dominant bacterium in the mats. In order to assess the bioremediation potential of the isolate for metal removal from contaminated water, an initial scan of tolerance to various metals was performed (data not shown). Based on these results, a selection of Cr(VI) was made for further studies in metal removal rates from contaminated water. Although Cr(VI) was the only metal included in these studies, metal tolerances indicate that this microbe may...
be important in the bioremediation of other metals as well. The identification of this bacterium as a putative new member of non-sulfur purple bacteria was based on its morphological, physiological characteristics, and DNA analyses. These results suggest that this isolate is a mesothermophilic gram-negative rod-shaped bacterium that shares general characteristics with the *Rhodopseudomonas* a genus of *Rhodospirillaceae* family. Cellular and morphological similarities of the isolate to the previously described species in the genus *Rhodopseudomonas* are: the presence of a lamellar intracytoplasmic membrane system, the bactochlorophyll a and reproduction by budding [1–3]. Electron micrographs of the isolate also exhibited these characteristics (Figs. 1 and 2).

The isolate did not grow on sulfide-containing medium and failed to form intracellular sulfur globules that distinguished this strain from the sulfur purple bacteria. The general identification of the isolate as a *Rhodopseudomonas* spp. is consistent with results from ATCC.

The RAPD-PCR profile of the isolate had a pattern that was clearly distinguishable from other known *Rhodopseudomonas* species. The physiological and morphological properties of the isolate are also different from those of the other known *Rhodopseudomonas* species [2], which was consistent with the RAPD-PCR results.

Comparison of the partial 16S-rDNA sequence (407 bp) of this isolate with the sequences found in the GenBank database yielded 97% homology to *Nitrobacter* and several *Bradyrhizobium* and *Rhodopseudomonas palustris* strains. Using SEQUENCE-MATCH from the Ribosomal Database Project II we determined that this isolate belongs to the purple bacteria, alpha subdivision of *proteobacteria* based on the fact that sequence homology corresponded with the *rhizobium-agrobacterium* group, *bradyrhizobium* subgroup. This study and other phylogenetic studies using 16S-rRNA sequence homologies [5,6] have demonstrated that species *Rhodopseudomonas palustris* is more closely related to some chemotrophic taxa, such as *Bradyrhizobium* species, *Nitrobacter* winogradskyi and *Afipia felis* than to any of the other *Rhodopseudomonas* species. Although data obtained from this study clearly provide reliable evidence for close relationships between this isolate and *Rhodopseudomonas palustris*, it would be unduly optimistic to hope that analysis of 407 nucleotide positions of 16S-rDNA, many of which are invariant, could allow an unambiguous reconstruction of all deeper branches in the phylogeny of this isolate to already known *R*. species. However, our results show that the isolate, *Bradyrhizobium* spp. and *Rhodopseudomonas palustris* belong to the same lineage, and are distinct from all other *Rhodopseudomonas* species. Based on the results of this study, we suggest that this isolate is an unidentified strain of *Rhodopseudomonas* species. The bioremediation properties of this isolate will be presented in another report.

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References

