Short Communication

Characterization of thermotolerant purple nonsulfur bacteria isolated from hot-spring *Chloroflexus* mats and the reclassification of “*Rhodopseudomonas cryptolactis*” Stadtwald-Demchick et al. 1990 as *Rhodoplanes cryptolactis* nom. rev., comb. nov.

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Geothermal hot springs are common sources not only of thermophilic anoxygenic phototrophic bacteria and cyanobacteria (Castenholz and Pierson, 1995; Hanada, 2003; Madigan, 2003) but also of thermotolerant phototrophic purple nonsulfur (PPNS) bacteria (Favinger et al., 1989; Gorlenko et al., 1985; Hisada et al., 2007; Namsaraev et al., 2003; Resnick and Madigan, 1989; Stadtwald-Demchick et al., 1990; Yurkov and Gorlenko, 1992). One of the best characterized thermotolerant PPNS bacteria is “*Rhodopseudomonas cryptolactis*” strain DSM 9987^T (Stadtwald-Demchick et al., 1990), which was isolated from Thermopolis Hot Springs, Wyoming, USA. During the course of our research on the biodiversity of PPNS bacteria in hot spring microbial mats, we isolated thermotolerant PPNS bacteria phylogenetically affiliated with the genus *Rhodoplanes* with “*Rps. cryptolactis*” as their closest relative (Hisada et al., 2007). In this paper, we propose the name *Rhodoplanes cryptolactis* nom. rev., comb. nov. to accommodate “*Rhodopseudomonas cryptolactis*” strain DSM 9987^T and these novel ther-

motolerant PPNS bacteria.

The new thermotolerant PPNS bacteria were isolated from orange-brown colored *Chloroflexus* mats that had developed in a hot spring stream (65°C, pH 8.3) of Nakanoyu, the Nagano prefecture, Japan, through the enrichment with PE medium (Hanada et al., 1995) and SAYS medium (Okubo et al., 2005) at 50°C and then at 37°C (Hisada et al., 2007). The enrichment culture at 50°C contained *Chloroflexus* exclusively, but a shift of the incubation temperature to 37°C resulted in the overgrowth pink-colored PPNS bacteria. From these pink cultures, the PPNS strains were isolated by the agar shake dilution method and repeated streaking of agar plates (Hisada et al., 2007).

Seven strains of the thermotolerant PPNS bacteria thus isolated (designated strains TUT3521 to TUT3527) were characterized in comparison with the authentic strains, *Rhodoplanes (Rpl.) elegans* strain AS130^T(=JCM 9224^T), *Rpl. roseus* strain DSM 5909^T, and “*Rhodopseudomonas (Rps.) cryptolactis*” strain DSM 9987^T. The strains with DSM numbers were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *Rpl. elegans* strain AS130^T (Hiraishi and Ueda, 1994) was isolated from pond water by one of us (A.H.). For cultivation of the test organisms, PYS (Hiraishi and Ueda, 1994) medium, which contained 20

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mm pyruvate (filter sterilized) as the sole carbon source, was used. For growth of "*Rps. cryptolactis*" strain DSM 9987^T and the new thermotolerant isolates, PYS medium was modified by supplementation with 20 µg of vitamin B₁₂, 10 µg of nicotinic acid, 3 µg of *p*-aminobenzoic acid, and 0.5 g of Na₂S₂O₃ · 5H₂O (per liter) (designated PYSV medium). All media were adjusted to pH 6.8. The cultivation was performed anaerobically under incandescent illumination (10 W m⁻²). Unless otherwise noted, the temperature of cultivation was 30°C for the authentic *Rhodoplanes* strains and 40°C for "*Rps. cryptolactis*" strain DSM 9987^T and the new isolates. Morphology and related properties were studied under an Olympus phase-contrast microscope and a JEOL transmission electron microscope. The photosynthetic membrane arrangement of cells was determined by ultrathin-section electron microscopy as described previously (Matsuzawa et al., 2000). Absorption spectra of cell extracts were measured with a Shimadzu Biospec 1600 spectrophotometer. Anaerobic growth by nitrate respiration in darkness was determined in screw-capped test tubes completely filled with PYSV medium supplemented with 20 mM KNO₃. N₂ gas production by complete denitrification was observed in these test tubes with Durham tubes. Photoassimilation of organic substrates was determined in screw-capped test tubes containing PYSV medium in which pyruvate was replaced with an organic carbon source. Photolithotrophic growth was determined in PYSV medium in which pyruvate was replaced with either 20% H₂ (v/v in headspace), 0.5 mM Na₂S, or 0.5 mM Na₂S₂O₃ as the electron donor and 0.1% NaHCO₃ (w/v) (filter sterilized) as the carbon source. Aerobic chemolithotrophic growth with thiosulfate was determined in the same medium as noted above. Nitrogen source utilization was determined by replacing (NH₄)₂SO₄ with different nitrogen sources at a concentration of 0.1% (w/v). Diazotrophic growth was determined in PYSV medium in which (NH₄)₂SO₄ was replaced with glutamine as the nitrogen source, and H₂ gas production in test tubes with Durham tubes was judged as being positive for nitrogen fixation. Growth was monitored turbidimetrically at 660 nm, and the final reading was taken after 2 weeks of incubation. All other physiological and biochemical tests were performed as described previously (Hiraishi and Ueda, 1994). Whole-cell fatty acids were analyzed by gas-liquid chromatography of their methyl ester derivatives as described previously (Hiraishi et al., 2002). Quinones

were extracted with a chloroform-methanol mixture, purified by TLC, and analyzed by HPLC as described previously (Hiraishi and Hoshino, 1984). A phylogenetic analysis was performed based on 16S rRNA gene sequences determined previously (Hisada et al., 2007) and retrieved from the DDBJ/EMBL/GenBank database. Multiple alignment of sequence, calculation of the corrected evolutionary distance (Kimura, 1980), and construction of a neighbor-joining phylogenetic tree (Saitou and Nei, 1987) were performed using the CLUSTAL W program ver. 1.83 (Thompson et al., 1994). The topology of the tree was evaluated by bootstrapping with 1,000 resamplings (Felsenstein, 1985). Genomic DNA was extracted and purified by the method of Marmur (1961), and its base composition was determined by the HPLC method with external nucleotide standards (Mesbah et al., 1989). DNA-DNA hybridization studies were performed by the dot-blot hybridization method with alkaline phosphatase labeling and chemiluminescence detection using an Amersham-Pharmacia AlkalPhos kit. Detailed information on the DNA-DNA hybridization procedure has been given previously (Hiraishi et al., 2002).

The 7 isolates of the thermotolerant PPNS bacteria had Gram-negative, motile, rod-shaped cells measuring 1 µm in width and 2–4 µm in length. Motile cells had single polar or two subpolar flagella. Cells divided asymmetrically by budding and formed rosette-like clusters in older cultures. The doubling time for cells optimally growing in PYSV medium was ca. 5 h. As shown in Fig. 1, electron microscopy of ultrathin sections revealed that phototrophically grown cells formed intracytoplasmic membranes of the lamellar type typical of PPNS bacteria belonging to the order *Rhizobiales* (Imhoff et al., 2005). Cell-free extracts from cultures grown at a low light intensity (2 W m⁻²) had absorption maxima at 800 and 822–825 nm and a lower peak at 875–878 nm in the near infrared region, whereas those from high-light-grown cultures (20 W m⁻²) showed major peaks at 800 and 857 nm and a lower peak at 822–823 nm (Fig. 2). These spectroscopic features are similar to those found in "*Rps. cryptolactis*" strain DSM 9987^T. As common properties of *Rhodoplanes* species (Hiraishi and Ueda, 1994), the isolates were able to grow not only aerobically at full atmospheric oxygen tension but also anaerobically in darkness with nitrate as the terminal electron acceptor. Nitrate-respiring cells produced nitrogen gas, thereby confirming their capacity for complete denitrification.

Fig. 1. Electron micrograph of an ultrathin section of strain TUT3521, showing the intracytoplasmic membranes (indicated by an arrow).

Fig. 2. Absorption spectrum of the cell extract of strain TUT3521.

Solid and dotted lines show the spectrum obtained from cells grown at a light intensity of 2 W m^{-2} and 20 W m^{-2} , respectively.

Anaerobic denitrifying growth in darkness was also observed for “*Rps. cryptolactis*” strain DSM 9987^T. Photolithotrophic growth occurred with H_2 but not with sulfide or thiosulfate as the electron donor. Aerobic chemolithotrophy with thiosulfate could not be demonstrated. The ability to fix nitrogen was confirmed by observing H_2 gas production with glutamine as the sole nitrogen source. The 7 isolates resembled “*Rps. cryptolactis*” strain DSM 9987^T in all other physiological characteristics tested (see the species description below).

The new isolates and “*Rps. cryptolactis*” strain DSM 9987^T also shared chemotaxonomic characteristics. Whole-cell fatty acid analyses revealed that $\text{C}_{18:1}\omega 7c$ predominated (73–77%) in all isolates as well as in strain DSM 9987^T (Table 1). Significant proportions of

Table 1. Cellular fatty acid profiles of the hot spring isolate and related strains.

Component	Composition (%)			
	Strain TUT3521	“ <i>Rps.</i> <i>cryptolactis</i> ” DSM 9987 ^T	<i>Rpl.elegans</i> AS130 ^T	<i>Rpl.roseus</i> DSM 5909 ^T
$\text{C}_{16:0}$	14.9	18.1	15.8	17.8
$\text{C}_{16:1}\omega 7c$ alcohol	t ^a	—	0.7	t
$\text{C}_{16:1}\omega 7c$	t	t	1.6	4.0
$\text{C}_{18:0}$	4.6	3.5	3.8	t
$\text{C}_{18:1}\omega 7c$	77.4	73.3	78.1	74.2
11Methyl $\text{C}_{18:1}\omega 7c$	3.2	3.1	t	2.8
$\text{C}_{19:0}$ cyclo $\omega 8c$	t	2.1	0	0
anteiso- $\text{C}_{19:0}$	t	t	0	t
$\text{C}_{16:0}$ 3-OH	t	t	t	1.3

^aTrace amounts (<0.5%).

$\text{C}_{16:0}$ (14–18%) were also detected. The isolates and “*Rps. cryptolactis*” strain DSM 9987^T contained both ubiquinone-10 and rhodoquinone-10 as primary quinone components. Menaquinones were absent.

The isolates and “*Rps. cryptolactis*” strain DSM 9987^T had identical 16S rRNA gene sequences, as previously reported (Hisada et al., 2007). They showed a sequence similarity level of 98.9% to *Rpl. elegans* strain AS130^T and of 98.2% to *Rpl. roseus* strain DSM 5909^T. As shown in Fig. 3, a 16S rRNA gene-based phylogenetic analysis showed that the 7 isolates and “*Rps. cryptolactis*” strain DSM 9987^T formed a distinct cluster within the genus *Rhodoplanes*. Genomic DNA-DNA pairing studies showed that one of the new isolates, strain TUT3521, and “*Rps. cryptolactis*” strain DSM 9987^T were highly related to each other at a hybridization level of 80–90%. Strain TUT3521 also had hybridization levels of 50 and 45% to *Rpl. roseus* strain DSM 5909^T and *Rpl. elegans* strain AS130^T, respectively. The G+C content of the genomic DNA of the 7 isolates ranged from 68.8 to 69.0%, resembling that of “*Rps. cryptolactis*” strain DSM 9987^T.

As reported herein and previously (Hisada et al., 2007), it is clear that the new thermotolerant strains together with “*Rps. cryptolactis*” DSM 9987^T represent a single species within the genus *Rhodoplanes* that is genotypically and phenotypically distinguishable from the previously established species of this genus, *Rpl. elegans* and *Rpl. roseus*. “*Rps. cryptolactis*” DSM 9987^T was originally isolated from a hot spring in the

Fig. 3. Neighbor-joining phylogenetic tree of the isolates and authentic *Rhodoplanes* strains based on 16S rRNA gene sequences.

The 16S rRNA gene sequence of *Rhodopseudomonas palustris* strain ATCC 17001^T was used as an outgroup to root the tree. The database accession numbers are given in parentheses following the strain names. Bootstrap confidence values expressed as the percentage of 1,000 bootstrap trials are given at branched points. Scale bar = 1% sequence divergence (K_{nuc}).

USA and described as a new species of the genus *Rhodopseudomonas* in 1989 (Stadtwald-Demchick et al., 1990). Since then, however, the species name has not been validated. Also, in light of the current taxonomic criteria for the genus *Rhodopseudomonas* and other genera of PPNS bacteria, "*Rps. cryptolactis*" is apparently a misclassified name. Therefore, we propose *Rhodoplanes cryptolactis* nom. rev., comb. nov. to accommodate our new isolates and "*Rps. cryptolactis*" strain DSM 9987^T. Diagnostic characteristics of *Rpl. cryptolactis* and other *Rhodoplanes* species are shown in Table 2. One of the new isolates, *Rpl. cryptolactis* strain TUT3521, has been deposited with the NITE Biological Resource Center (NBRC, Kisarazu, Japan) as strain NBRC 102048.

Description of *Rhodoplanes cryptolactis* nom. rev., comb. nov.

Rhodoplanes cryptolactis (cryp.to'lac.tis. Gr. adj. *cryptos* hidden; L. ferm. n. *lactes* milk; M.L. ferm. adj. *cryptolactis* hidden of milk).

Cells are Gram-negative rods measuring 1.0 μm wide and 2.5–4.0 μm long, and multiply by budding. Motile by single polar or two subpolar flagella. Rosette formation occurs in older cultures. Intracytoplasmic photosynthetic membranes are of the lamellar type parallel to the cytoplasmic membrane. Bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series are

Table 2. Differential characteristics of *Rpl. cryptolactis* nom. rev., comb. nov. and other *Rhodoplanes* species.^a

Characteristic	<i>Rpl. cryptolactis</i> nom. rev., comb. nov.	<i>Rpl. roseus</i>	<i>Rpl. elegans</i>
Cell length (μm)	2.5–4.0	2.0–2.5	2.0–3.0
Type of budding	Tube	Sessile	Tube
Rosette formation	+	–	+
In vivo absorption maxima (nm) in infrared region ^b	801–802, 822–823, 857	801, 854	799, 855
Temperature ($^{\circ}\text{C}$) for growth:			
Optimum	40	30	30
Upper limit	45–46	38	40
Sulfate assimilation		+	+
Vitamins required	Niacin, <i>p</i> -ABA, B ₁₂	Niacin	<i>p</i> -ABA, thiamine
G+C (mol%)(HPLC)	68.8–69.0	66.8	69.6–69.7
Habitat	Hot spring	Freshwater	Freshwater

^a Information based on Hiraishi and Ueda (1994), Stadtwald-Demchick et al. (1990), and this study.

^b Data obtained with cultures grown under high-light conditions.

present. Phototrophically grown cells have absorption maxima at 801–802, 822–825, and 877–878 nm (low light conditions) and at 801–802, 822–823, and 857 nm (high light conditions) in the near infrared region. Anaerobic photoorganotrophy is the preferred mode of growth. Aerobic growth in darkness occurs at full atmospheric oxygen tension. Anaerobic growth in darkness with nitrate as the terminal electron acceptor is also possible; denitrification positive. Anaerobic phototrophic cultures are pink to red and aerobic chemotrophic cultures are faint pink or colorless. Temperature range for growth is 25–46 $^{\circ}\text{C}$ (optimum 40 $^{\circ}\text{C}$). The pH range for growth is 6.4–8.5 (optimum pH 6.8–7.2). Little or no growth occurs in the presence of 1% NaCl and more. Vitamin B₁₂, nicotinic acid, and *p*-amino benzoic acid are required as growth factors. Photolithotrophic growth occurs with H₂ but not with sulfide or thiosulfate as the electron donor. Usable carbon sources for phototrophic growth are pyruvate, acetate, malate, succinate, butyrate, and yeast extract. Propionate is used by some strains. Not utilized are citrate, L-arabinose, glucose, fructose, lactate, benzoate, tartrate, glutamate, glutamine, alanine, aspar-

tate, and arginine. Diazotrophic growth occurs. Glutamine and urea are utilized as the nitrogen source. Thiosulfate but not sulfate is assimilated as the sulfur source. The predominant component of cellular fatty acids is C_{18:1}ω7c. The major quinones are ubiquinone-10 and rhodoquinone-10. The G+C content of genomic DNA ranges from 68.8 to 69.0 mol%. Habitats are geothermal hot springs. The type strain is strain DSM 9987 (=ATCC 49414).

The database accession number for the 16S rRNA gene sequence of *Rhodoplanes cryptolactis* strain DSM 9987^T is AB087718.

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