

## Molecular Level Relationships of Purple Nonsulfur Bacteria and their Relatives

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DNA-DNA hybridization by kinetic method was carried out between species of purple nonsulfur photosynthetic bacteria and nonphotosynthetic bacteria. The degrees of homology percent were shown to be low (2~35 D%) with the exception of high homology % (72~88 D%) for strains within a species and between *Rhodobacter capsulatus* and *Rhodopseudomonas blastica*. The D% between the purple nonsulfur photosynthetic bacteria, *Rhodopseudomonas palustris*, and nonphotosynthetic bacteria, *Pseudomonas aeruginosa* ATCC 27853 or *Bradyrhizobium japonicum* were a little higher (26~33 D%) than the D% between any other photosynthetic bacteria. The homology % between *Rhodopseudomonas blastica* and *Rhodobacter capsulatus* was 72 D%, which showed genetic relationship.

**KEY WORDS** □ purple nonsulfur photosynthetic bacteria, nonphotosynthetic bacteria, DNA-DNA hybridization

The purple nonsulfur photosynthetic bacteria are classified as Rhodospirillaceae based on morphological, physiological and biochemical characteristics (24, 28, 29). But according to cytochrome pattern analysis (1, 2, 3, 10), 16S rRNA oligonucleotide catalogue (7, 12, 20, 25, 33) and DNA-rRNA hybridization (7, 33), it is noticed that some of the purple photosynthetic bacteria have more relatedness with nonphotosynthetic bacteria than with any other. Because of the difficulty in identifying evolutionary differences, the International Committee on Systematic Bacteriology agreed to use the name "purple bacteria and their relatives" rather than Rhodospirillaceae, and renamed it "Proteobacteria" at the level of class in 1988 (26), because many of them did not belong to photosynthetic bacteria.

DNA-rRNA hybridization showed more relatedness between *Rhodopseudomonas palustris* and *Rhizobium*, and between *Rhodopseudomonas palustris* and *Bradyrhizobium* than with any other photosynthetic bacteria (19). 16S rRNA catalogue and cytochrome c analysis patterns showed that *Rhodocyclus gelatinosus* also is more related to the nonphotosynthetic bacteria. *Pseudomonas* and *Sphaerotilus natans* (1, 2, 12).

The outstanding attribute of the major phylogenetic branches within Proteobacteria is the diversity of shape and physiology. With the object of forming the basis of discrimination at the species level of Proteobacteria, we report DNA-

DNA relatedness between species of nonsulfur photosynthetic and nonphotosynthetic bacteria according to kinetic method.

### MATERIALS AND METHODS

#### Bacterial strains and culture conditions

The strains and culture media used in this study are listed in Table 1. The organisms were cultivated anaerobically in the light (under 2,000 lux incandescent light) at 28~30°C in completely filled bottles. Nonphotosynthetic bacteria were shaken in the dark.

#### Isolation and purification of DNA

The cells were harvested after exponential phase growth and DNA was isolated by the method of Marmur (22), Hoyer and McCullough (14) and Staley and Colwell (27). Concentration and the purity of the DNA were determined spectrophotometrically (Uvikon 930 UV/visible spectrophotometer equipped with thermostat).

#### Fragmentation and size determination of DNA

DNA was sonicated (Ultrasonic-homogenizer Lab-sonic 2000 B, Braun) to about 180 to 1000 base pairs according to Brenner *et al.* (4, 5) and Gebers *et al.* (11). To determine this size, agarose gel electrophoresis (1.2%) was carried out with a 1 kb ladder used as a marker (21).

#### T<sub>m</sub> and G+C content of DNA

The mole percentage of G+C was determined by the thermal denaturation procedure of DNA

**Table 1.** Strains and culture media used in this study.

Strains	Culture media
<i>Rhodospirillum rubrum</i> DSM 467	DSM 27
<i>Rhodopseudomonas palustris</i> DSM 123	DSM 27
<i>Rhodopseudomonas palustris</i> ATCC 17003	DSM 27
<i>Rhodopseudomonas blastica</i> ATCC 33485	DSM 27
<i>Rhodomicrobium vannielii</i> DSM 162	DSM 26
<i>Rhodocyclus gelatinosus</i> ATCC 17011	DSM 27
<i>Rhodocyclus tenuis</i> ATCC 25093	ATCC 550
<i>Rhodobacter capsulatus</i> DSM 1710	DSM 27
<i>Rhizobium leguminosarum</i> ATCC 10004	<i>Rhizobium</i> medium
<i>Bradyrhizobium japonicum</i> ATCC 10324	<i>Rhizobium</i> medium
<i>Sphaerotilus natans</i> ATCC 15291	<i>Sphaerotilus</i> medium
<i>Pseudomonas acidovorans</i> ATCC 15668	Nutrient broth
<i>Pseudomonas acidovorans</i> ATCC 11299a	Nutrient broth
<i>Pseudomonas aeruginosa</i> NCTC 10332	Nutrient broth

DSM, Deutsche Sammlung für Mikroorganismen und Zellkulturen; ATCC, American Type Culture Collection.

**Table 2.**  $T_m$  and G+C% of DNA from strains.

Strains	$T_m$ (°C)	G+C%	Standard
<i>Rs. rubrum</i> DSM 467	82.6	65.4	65.4
<i>Rps. palustris</i> DSM 123	82.8	65.8	65.8
<i>Rps. palustris</i> ATCC 17003	82.7	65.6	
<i>Rps. blastica</i> ATCC 33485	82.5	65.3	
<i>Rc. gelatinosus</i> ATCC 17011	85.2	70.8	71.4
<i>Rc. tenuis</i> ATCC 25093	82.35	64.9	64.8
<i>Rb. capsulatus</i> DSM 1710	83.7	67.7	69.6
<i>Rz. leguminosarum</i> ATCC 10004	81.3	62.7	59-63
<i>B. japonicum</i> ATCC 10324	81.4	63.0	
<i>P. aeruginosa</i> NCTC 10332	83.8	67.9	67.2
<i>P. acidovorans</i> ATCC 11299a	83.5	67.3	67.0

$T_m$  was determined in 0.1 SSC and G+C% was calculated to formula of  $\%(G+C)=2.08T_m-106.4$ . DNA concentration was 40  $\mu\text{g/ml}$  and temperature was raised about 0.5°C per min.

in 0.1×SSC and calculated according to Owen and Pitcher (23).

#### DNA-DNA hybridization

DNA concentration for hybridization carried on with either 32  $\mu\text{g/ml}$  or 50  $\mu\text{g/ml}$  of 30% DMSO (dimethyl sulfoxide) in 2×SSC according to Escara and Hutton (9).

For denaturation, DNA was heated for 10 min at 100°C according to De Ley *et al.* (6) and Huss *et al.* (15, 16, 17) and for 5 min with DMSO in accordance with Escara and Hutton (9). To determine the optimal renaturation rate, several different temperatures were tested. The optimal renaturation temperature ( $T_{or}$ ) in 2×SSC was compared to the  $T_{or}$  of Gillis formula (13).

Renaturation rates,  $V'$ , were determined as decrease in absorbance/min( $\Delta A/t$ ) at 260 nm over 20 min, and were measured 10 min after the start of the reaction.

DNA-DNA homologies were carried out in a spectrophotometer equipped with a thermostat

and calculated according to De Ley *et al.* (6).

## RESULTS

#### DNA purification

Purified DNA for estimation of homologies should reveal ratios of absorbance at 260 to 280 nm greater than 1.87 and at 260 to 230 nm greater than 1.92.

#### DNA fragmentation

For renaturation experiments, DNA was sheared by ultrasonification either for 7 min 30 sec in 3 ml of 40  $\mu\text{g/ml}$  or 12 min 30 sec in 10 ml of 100  $\mu\text{g/ml}$ . Fragmented size were 200~1,100 base and 200~1,400 base. The G+C contents of all strains showed between 62.7 and 67.9 mol% except *Rhodocyclus gelatinosus* (Table 2).

#### Temperature of renaturation

Optimal renaturation temperature in 2×SSC was 2°C lower than calculated by Gillis formula using same species, but 75°C in good agreement

**Table 3.** The degree of binding (D%) of 50 µg/ml (in 2×SSC/30% DMSO) DNA preparations.

	rub	pal	pal*	bla	gel	ten	cap	leg	jap	nat	aci	aci*	aer	aer*
rub	100													
pal		100												
pal*		74	100											
bla		13		100										
gel	13	7	16		100									
ten	22	17			30	100								
cap		14	16	72	21	23	100							
leg	NA	2			5	NA	35	100						
jap		32	33					27	100					
nat					15					100				
aci	3	12	5	27	17	14	8	NA		NA	100			
aci*											88	100		
aer	14	16			20	23	21	NA			19	19	100	
aer*		26	29	8	22		12		29		32		85	100

rub, *Rs. rubrum* DSM 467; pal, *Rps. palustris* DSM 123; pal\*, *Rps. palustris* ATCC 17003; bla, *Rps. blastica* ATCC 33485; gel, *Rc. gelatinosus* ATCC 17011; ten, *Rc. tenuis* ATCC 25093; cap, *Rb. capsulatus* DSM 1710; leg, *Rz. leguminosarum* ATCC 10004; jap, *B. japonicum* ATCC 10324; nat, *S. natans* ATCC 15291; aci, *P. acidovorans* ATCC 15668; aci\*, *P. acidovorans* ATCC 11299a; aer, *P. aeruginosa* NCTC 10332; aer\*, *P. aeruginosa* ATCC 27853.

NA, data not available.

in a mixture, therefore all renaturation rates were determined at 75°C except that of *Rhizobium leguminosarum*, which was a G+C% lower than others, and determined at 60°C instead of 75°C.

Effect of 30% DMSO was an increased renaturation rate. Temperature of renaturation was lowered about 10~15°C with addition of DMSO.

#### DNA-DNA hybridization

Heat denatured DNA was renatured over 20 min after 10 min of initiation time and the renaturation rate was expressed as decrease in absorbance at 260 nm/min ( $V' = \Delta \text{ABS}/\text{min}$ ) which was the slope of the reassociation curve obtained by regression analysis. Each value was an average outcome of 3 repetitions. For the DNA homology relatedness, renaturation rate was found by the method of De Ley *et al.* (6), and the percentage hybridization was then calculated from the formula. The results are shown in Table 3.

The degree of homology percent with the exception of high homology % (72~88%) for strains with in a species and between *Rhodobacter capsulatus* and *Rhodospseudomonas blastica* was shown to be low D% (2~35%). The D% between the purple nonsulfur photosynthetic bacteria, strains of *Rhodospseudomonas palustris*, and the nonphotosynthetic bacteria, *Pseudomonas aeruginosa* ATCC 27853 and *Bradyrhizobium japonicum* showed higher D% (26~33%) than homology % between any other photosynthetic bacteria. Other references showed 30% between *Rhodocyclus tenuis* and *Rhodocyclus gelatinosus* and 35% between *Rhizobium leguminosarum* and

*Rhodobacter capsulatus*. Table 3 shows total relationships among these bacteria.

## DISCUSSION

The optical technique of DNA-DNA hybridization was used to study the genetic relationship of 11 species, 15 strains belonging to the class Proteobacteria.

G+C% by the thermal denaturation method was determined in 0.1×SSC at 80°C in order to decrease error caused by mechanical temperature loss with 1×SSC in which T<sub>m</sub> showed above 90 °C. These species had G+C contents of 62.7 to 70.8 mol%, which agrees well with standard. There was no large differences in G+C content except 70.8 mol%.

Vauterin *et al.* (30) and Wallbanks *et al.* (31) noticed that the intra-special (50% homologies), interspecial (28~36% homologies) and intergeneric (10~16%) levels of similarity between DNA nucleotide sequences were consistent with the conventional criteria of relationship based on the comparison of bacterial genomes. A DNA homology value of 60 to 70% is the generally accepted minimum value for strain within a species. Huss *et al.* (16) noticed that values below 30 D% with optical method did not show any relationship genetically, because this method had disadvantages of high background values, but optical method was useful, because no labeled DNA was required.

The degree of homology % among tested strains were in agreement with Vauterin *et al.* (30). All

hybridization was performed in 2×SSC containing 30% DMSO and 50 µg/ml of DNA according to Huss *et al.* (16, 17). The addition of 30% DMSO in the hybridization solution increased the renaturation rate and decreased the *T<sub>m</sub>* by 10~15°C.

We also tested the DNA hybridization kinetics in 2×SSC containing only the 32 µg/ml DNA by Huss *et al.* (15). D% between *Pseudomonas acidovorans* strains 82 D%, which was almost the same value obtained when 50 µg/ml of DNA and 30% DMSO were used in hybridization. The D% between *Rhodobacter capsulatus* DSM 1710 and ATCC 17016 was 93 D%. According to Jarvis *et al.* (19), their data showed for the first time that the rRNA cistrons of *Bradyrhizobium* were highly related to those of *Rhodopseudomonas palustris*. On this basis, they suggested that photosynthetic ancestor of *Bradyrhizobium* may have been a *Rhodopseudomonas* and that a phylogenetic classification should group these genera together. The results of DNA homology studies showed more relatedness between *Rhodopseudomonas palustris* and *Bradyrhizobium japonicum* (32 and 33 D%) than between photosynthetic bacteria themselves (Table 3). But there was no remarkable DNA homology degree between *Rhodopseudomonas palustris* and *Rhizobium leguminosarum*. However, Gibson *et al.* (12) noticed more relatedness to each other (between *Rhodopseudomonas palustris* and *Rhizobium leguminosarum*) than to any other purple photosynthetic bacteria, according to the analysis of cytochrome *c* sequence and 16S rRNA catalogue. The degree of DNA homology percent was shown more relatedness between *Rhodobacter capsulatus* and *Rhizobium leguminosarum* (35 D%) than among others.

*Sphaerotilus natans* and *Rhodocyclus gelatinosus* are physiologically different, but in terms of 16S rRNA similarity according to Woese *et al.* (33) they belong to β-1 subgroup with *Pseudomonas acidovorans* but they showed no relations of chromosomal DNA homology (Table 3).

The D% between *Rhodopseudomonas blastica* and *Rhodobacter capsulatus* was 72%. *Rhodopseudomonas blastica* is placed on another branch of this superfamily together with the species included in the new genus *Rhodobacter* by the hybridization studies of DNA and rRNA. Because of its morphological properties and since other conflicting results are lacking, *Rhodopseudomonas blastica* is retained in the genus *Rhodopseudomonas* (18). Eckersley and Dow (8), morphologically and with respect to the mode of growth, *Rhodopseudomonas blastica* resembles the budding *Rhodopseudomonas* species of group 3. However, the simplicity of its cell cycle, its photopigments and its physiology suggests relatedness to group 1. *Rhodopseudomonas blastica* and *Rhodobacter*

*capsulatus* may be more related than currently classified.

Finally, as recommended by other researchers in this field (19, 32), we believe that several different methods and a wide range of bacterial strains should be applied before reach to the conclusion.

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**초 록: 홍색 비황박테리아와 그들의 연관군 사이의 분자수준의 연관관계**

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광합성 세균과 비광합성 세균에 속하는 종들 사이의 유연관계를 파악하기 위해 DNA 혼성화 방법을 실시하였다. 혼성화도는 종내 균주들 사이와 *Rhodobacter capsulatus*와 *Rhodopseudomonas blastica* 사이를(72~88%) 제외하고는 전체적으로 낮게 나타났다(2~35%). 광합성 세균 *Rhodopseudomonas palustris*와 비광합성 세균 *Pseudomonas aeruginosa* ATCC 27853, *Bradyrhizobium japonicum* 사이의 D%는 광합성 세균 사이의 D%보다 약간 높게 나타났다(26~33%). *Rhodopseudomonas blastica*와 *Rhodobacter capsulatus* 사이의 D%는 72%로 유전적 유연관계가 매우 높은 것으로 나타났다.