Lipopolysaccharide and porin of *Roseobacter denitrificans*, confirming its phylogenetic relationship to the α-3 subgroup of Proteobacteria

Uwe Neumann,1 Hubert Mayer,2 Emile Schiltz,3 Roland Benz4 and Jürgen Weckesser1

Author for correspondence: Jürgen Weckesser. Tel: +49 761 203 2638. Fax: +49 761 203 2647.

---

**INTRODUCTION**

Aerobic photosynthetic bacteria which contain bacteriochlorophyll *a* have been found in different ecological niches (Shiba et al., 1991; Fuerst et al., 1993; Yurkov & van Gemerden, 1993). They are represented by several genera such as the marine *Erythrobacter* (Shiba & Simidu, 1982) and *Roseobacter* (Shiba, 1991), the freshwater *Erythromicrobium* (Yurkov et al., 1992), *Roseococcus* (Yurkov & Gorlenko, 1992) and *Porphyrobacter* (Fuerst et al., 1993), as well as some species of *Methylbacterium* (Sató, 1987) and *Rhizobium* (Evans et al., 1990). All orange- and pink-pigmented obligately aerobic bacteria were originally included in one genus – *Erythrobacter* – with the type species *Erythrobacter longus* (Shiba & Simidu, 1982). Due to the taxonomic heterogeneity of this group, the pink-coloured strains were separated from *Erythrobacter* to form a new genus – *Roseobacter* – with the two representatives *Roseobacter denitrificans* and *Roseobacter litoralis* (formerly *Erythrobacter* sp. OCh 114 and OCh 149) (Shiba, 1991).

Within the phylogenetic tree, the aerobic photosynthetic bacteria containing bacteriochlorophyll *a* are combined with the anaerobic or facultatively anaerobic photosynthetic bacteria into the α-subgroup of Proteobacteria (Stackebrandt et al., 1988; Woese et al., 1984). By 16S rRNA sequence analysis it was demonstrated that the species of *Erythrobacter*, *Erythromicrobium* and *Porphyrobacter* belong to the α-4 and *Roseococcus* to the α-1 subclass, the latter being moderately related to *Rhodopila globiformis* and *Thiobacillus acidophilus*. *Roseobacter* belongs to the α-3 subclass (Fuerst et al., 1993), of which prominent members are *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rhodopseudomonas blastic*. Renaming of *Roseobacter denitrificans* as *Rhodobacter denitrificans* is suggested.

---

**Keywords**: *Roseobacter denitrificans*, lipopolysaccharide, porin, α-3 subgroup of Proteobacteria, phylogeny
**METHODS**

**Cultivation of bacteria.** Roseobacter denitrificans was taken from the strain collection of the Institut für Biologie II, Mikrobiologie, Freiburg i. Br., FRG. Mass cultures were grown aerobically in the dark at 32 °C according to Shioi (1986).

**Isolation of LPS.** LPS was isolated by the hot phenol-water procedure (Westphal & Jann, 1965) and sedimented three times by preparative ultracentrifugation in distilled water (105 000 g, 4 °C, 4 h). The extraction was repeated with the lyophilized pellet of the third centrifugation step. The final pellet, representing the LPS, was lyophilized.

**Analytical methods.** Sodium deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) was performed according to Komuro & Galanos (1988). Fatty acids were liberated by transesterification with conc. HCl/methanol (1:100, v/v) at 100 °C for 40 min for the isolation of the cell wall according to Schnaitman (1971). The oligomeric porin was obtained from the cell wall fraction by extraction with 2% (w/v) N,N-dimethyl-dodecylamidene-N-oxide (LDAO), 0.01% (v/v) 2-mercaptoethanol, 0.5 M NaCl, 10% (v/v) glycerol, 10 mM MgCl₂, 20 mM Tris/HCl, pH 8.0, at 38 °C for 70 min. After centrifugation (170 000 g, 4 °C, 60 min) the supernatant enriched with the porin was dialyzed against 0.08% (w/v) LDAO, 20 mM NaCl, 2 mM Na₃P₂O₇, 20 mM Tris/HCl, pH 8.0 (buffer A of the subsequent anion-exchange chromatography) for 15 h. Aliquots of the dialysed supernatant were applied on a Mono P HR 5/5 anion-exchange-chromatography column (Pharmacia). To remove photopigments the column was washed with 10 ml 1% (w/v) LDAO, 4% (w/v) betaine, 20 mM NaCl, 2 mM Na₃P₂O₇, 20 mM Tris/HCl, pH 7.8. Subsequently the column was eluted with a linear salt gradient of 0-0.2 M NaCl in 30 ml at a flow rate of 1 ml min⁻¹ with an FPLC system (Pharmacia). For further purification the porin obtained by this procedure was applied on a Superose 12 HR 10/30 gel-permeation-chromatography column (Pharmacia) and eluted with 0.08% (w/v) LDAO, 300 mM LiCl, 2 mM Na₃P₂O₇, 20 mM Tris/HCl, pH 7.8.

For determination of EDTA-sensitivity, porin purified by ion-exchange gel-permeation chromatography was incubated with SDS-PAGE sample buffer containing 2-10 mM EDTA for 30 min at room temperature. Subsequently a 0.1 M CaCl₂ solution was added to one half of the samples in a 1:1 ratio to see whether the dissociation into monomers was reversible. All samples were incubated for another 30 min at room temperature and finally applied directly onto SDS-PAGE (see below).

**SDS-PAGE.** Discontinuous SDS-PAGE was performed using 14% (w/v) acrylamide slab gels. Gels were stained with Coomassie R-250.

**N-terminal sequence determination of porin.** The N-terminal sequence of hexane/propan-2-ol-precipitated porin was determined by Edman degradation in a pulsed liquid protein sequencer model 477A (Applied Biosystems) equipped with a PTH-amino acid analyser type 120A (Applied Biosystems). Homology studies were made on the 12 N-terminal residues. The following amino acids were regarded as homologous: S,T / D,E / V,L,F,L,M,Y / A,P,G / K,R,H.

**RESULTS**

**Isolation of LPS and lipid A**

The LPS from Roseobacter denitrificans was isolated by two sequential phenol-water extractions and obtained from the water phase in a yield of 0.4% of cell dry mass. It was classified as rough (R)-type LPS, since it migrated as a single band in DOC-PAGE, when compared with several R-type LPSs of Salmonella minnesota, the LPS of Roseobacter denitrificans had a migration pattern similar to that of an R₈-mutant of Salmonella minnesota.

The lipid A moiety was split off from the LPS by mild acid hydrolysis (1%, v/v, acetic acid, 80 °C, 100 min). The yield of lipid A was 8% (w/w) of the LPS dry mass, that of the polysaccharide moiety 59% (w/w). Fatty acid analysis of lipid A revealed 3-hydroxydecanoic acid and 3-oxotetradecanoic (3-oxomyristic) acid in a molar ratio of 10:8 (the small amounts of 18:1 detected were considered as contamination). 3-Hydroxydecanoic acid was exclud-
Table 1. Chemical composition of the LPS, lipid A and polysaccharide fraction of Roseobacter denitrificans

<table>
<thead>
<tr>
<th>Component</th>
<th>Content (nmol mg⁻¹) in:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS</td>
<td>Lipid A</td>
</tr>
<tr>
<td>Ribose</td>
<td>16.9</td>
<td>ND</td>
</tr>
<tr>
<td>Arabinose</td>
<td>5.5</td>
<td>ND</td>
</tr>
<tr>
<td>Mannose</td>
<td>24.8</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose</td>
<td>39.2</td>
<td>ND</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>238.5</td>
<td>385.0</td>
</tr>
<tr>
<td>Glucosamine 6-phosphate</td>
<td>39.8</td>
<td>11.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>27.4</td>
<td>57.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.7</td>
<td>ND</td>
</tr>
<tr>
<td>Hexuronic acids</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>KDO</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Phosphate</td>
<td>ND</td>
<td>1330</td>
</tr>
<tr>
<td>3-Hydroxydecanoic acid</td>
<td>2.7</td>
<td>774.4</td>
</tr>
<tr>
<td>3-Oxotetradecanoic acid</td>
<td>4.0</td>
<td>953.1</td>
</tr>
</tbody>
</table>

ND, Not determined; −, not present; +, present, but not quantified.

Fig. 1. SDS-PAGE of porin released from outer membranes of Roseobacter denitrificans. Lanes: A, porin solubilized at 30 °C for 20 min; B, porin solubilized at 100 °C (5 min); S, molecular mass markers. The separating gel contained 14% acrylamide. Gels were run at a constant voltage of 150 V. Staining: Coomassie brilliant blue.

EDTA sensitivity and N-terminal amino acid sequence of porin

The porin of Roseobacter denitrificans migrated in SDS-PAGE as a single band with an apparent molecular mass of 88 kDa. The addition of 2 mM EDTA to the SDS-PAGE sample buffer caused a complete dissociation into monomers with an apparent molecular mass of 35 kDa at room temperature. Subsequent addition of CaCl₂ led to reaggregation into the oligomeric form.

The porin was purified by anion-exchange chromatography and gel-filtration chromatography; purity was checked by SDS-PAGE (Fig. 1) and N-terminal sequence determination. Gas-phase sequencing revealed the 12 N-terminal residues as follows: NH₂-Asp-Ile-Thr-Phe-Asp-Gly-Phe-Gly-Arg-Phe-Gly-Ile. Side-sequences were not observed. The N-terminal sequences of porins from other bacteria were compared for homology studies (Fig. 2). Significant sequence homology was found to porins of Rhodopseudomonas bfastica (91% identical or conservative exchanged amino acids), to Rhodobacter capsulatus (75% identical or conservative exchanged amino acids), and to Rhodobacter sphaeroides (83% identical or conservative exchanged amino acids). No sequence homology (less than 25%) was found to bacteria that do not belong to the α-3 subgroup of Proteobacteria except a 58% homology to Omp 1 from Neisseria species.

DISCUSSION

LPSs, especially their lipid A component, have been shown to reflect phylogenetic relationships between many Gram-negative bacteria (Weckesser & Mayer, 1988; Mayer et al., 1989). This has been found to be true also for traces of glucosamine 6-phosphate were also detected. The polysaccharide fraction (degraded polysaccharide) contained alanine and glycine but no glucosamine. As tested by high-voltage electrophoresis the LPS contained KDO. N-Acetylenuraminic acid was absent. In agreement with the R-character, neutral sugars were found only in small amounts. Two pentoses, ribose and arabinose, and two hexoses, glucose and mannose, but no heptoses were detected by GC-MS of the alditol acetate derivates. Uronic acids, examined by high-voltage electrophoresis and subsequent naphthoresorcinol staining, were not observed.
the α-3 subgroup of Proteobacteria, where the fatty acid pattern has proven to be a valuable phylogenetic marker. The lipid A of *Roseobacter denitrificans* contains the same fatty acid pattern as the other species of the α-3 subgroup. It includes the rare 3-oxotetradecanoic acid, which is amide-bound in both *Roseobacter denitrificans* and *Rhodobacter capsulatus* 37b4 (Krauss et al., 1989); in *Rhodobacter sphaeroides* 3-oxotetradecanoic acid is accompanied by amide-bound 3-hydroxytetradecanoic acid (Salimath et al., 1983). All these species have 3-hydroxydecanoic acid as the main ester-linked fatty acid (Weckesser et al., 1988). *Rhodopseudomonas blasitca*, recently described to belong also to the α-3 subgroup of Proteobacteria (Kawasaki et al., 1993), has the same characteristic fatty acid spectrum of amide-linked 3-oxotetradecanoic acid and ester-linked 3-hydroxydecanoic acid (Tegtmeyer et al., 1985). The main sugar component of the LPS of *Roseobacter denitrificans* is phosphorylated glucosamine, which originates from lipid A. All species of the α-3 subgroup so far studied have phosphorylated lipid A\(_{\text{GlcN}}\) (Weckesser & Mayer, 1988). In other subgroups of the α-group there are species possessing non-phosphorylated lipid A, such as lipid A\(_{\text{OAG}}\) (Weckesser & Mayer, 1988) in *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* or lipid A\(_{\text{GlcN}}\) in *Rhodobacter capsulatus* vonnielli (Mayer & Weckesser, 1984). The LPS of Roseobacter denitrificans – and that of *Rhodopseudomonas blasitca* – contain no heptoses, otherwise widespread as a characteristic core constituent of many Gram-negative bacteria. The lack of heptoses in the LPS is another characteristic of the bacteria of the α-3 subgroup of Proteobacteria (Weckesser et al., 1995).

The porin of *Roseobacter denitrificans* could be dissociated into monomers by EDTA-containing SDS-PAGE sample buffer at room temperature. This characteristic has been found so far only with porins of *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Paracoccus denitrificans*, all of which are members of the α-3 subgroup. Porins have recently been classified into ten groups according to their protein sequences (Schiltz et al., 1991). *Rhodobacter capsulatus* has so far been taken as the only member of the tenth group with homology values to the other groups that correspond those to the randomized sequence of *Rhodobacter capsulatus*. In the present study, homology of the *Roseobacter denitrificans* porin with those of all ten groups was tested. The comparison was performed with the 12 N-terminal amino acids, forming directly the first transmembrane strand. According to the model obtained with the porin of *Rhodobacter capsulatus* (Weiss et al., 1991), the β-strands spanning the outer membrane are believed to have rather conservative amino acid sequences. Good homology was found with porins of all species of the α-3 subgroup tested. Most interestingly, it was especially high (91 % identical or conservative exchanged amino acids) to the porin of *Rhodopseudomonas blasitca*, the structure of which is also known at atomic resolution (Kreusch et al., 1994). On the other hand, the homology values were less than 25 % identical or conservative exchanged amino acids to porins from bacteria not belonging to the α-3 subgroup, except to Omp 1 from *Neisseria meningitidis* and *Neisseria gonorrhoeae* (each having 58 % identical or conservative exchanged amino acids). *Neisseria* species are members of the β-3 subgroup of Proteobacteria (Woese, 1987), the LPS of the latter-mentioned species containing β-hydroxydecanoic acid among others; 3-oxotetradecanoic acid was not found in this earlier study, but only one column and no mass spectrometry was used for fatty acid identification (Stead et al., 1975).

The reported homology of porins within the α-3 subgroup of Proteobacteria may be taken as another indication of the phylogenetic relationship between *Roseobacter denitrificans* and the other bacteria of this subgroup. Together with the characteristics of the LPS, the homology of cytchrome c\(_{551}\) and the B\(_{370}\) polypeptides in comparison to those of *Rhodobacter capsulatus* (Okamura et al., 1987, Liebetanz et al., 1991), and the 16S rRNA data, it now seems to be proved that *Roseobacter denitrificans* belongs to the α-3 subgroup of Proteobacteria. Accepting the considerations for *Rhodopseudomonas blasitca* (Tegtmeyer et al., 1985; Kawasaki et al., 1993), renaming of *Roseobacter denitrificans* as *Rhodobacter denitrificans* would be the consequence.

**ACKNOWLEDGEMENTS**

The authors acknowledge detailed help by Dr W. Yurkov in refining the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB176, B9) and the Fonds der Chemischen Industrie.

**REFERENCES**


Received 15 November 1994; revised 11 April 1995; accepted 19 April 1995.