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

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***Roseobacter denitrificans* has rough (R)-type lipopolysaccharide, containing 2-keto-3-deoxyoctonate but no heptoses. Its lipid A has a glucosamine-containing, phosphorylated backbone. It contains the rare 3-oxotetradecanoic (3-oxomyristic) acid as the only amide-bound fatty acid and ester-bound 3-hydroxydecanoic acid, this pattern being characteristic for the α -3 subgroup of Proteobacteria. Treatment of the major outer-membrane protein (porin, apparent molecular mass 88 kDa) of *Roseobacter denitrificans* with EDTA (2 mM, 30 °C, 20 min) resulted in the dissociation of the oligomers into monomers (apparent molecular mass 35 kDa). EDTA-sensitive dissociation has so far been observed only within the α -3 subgroup of Proteobacteria. The 12 N-terminal amino acids of the monomers exhibit sequence homology with the porins of *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rhodopseudomonas blastica*. Renaming of *Roseobacter denitrificans* as *Rhodobacter denitrificans* is suggested.**

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

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 *Methylobacterium* (Sato, 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 *Rhodospira 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 *Paracoccus denitrificans*.

Data on lipopolysaccharide (LPS) and porin from aerobic photosynthetic bacteria have not hitherto been available. In this work, LPS and porin of *Roseobacter denitrificans* were characterized. The data confirm the assignment of

Abbreviations: DOC, sodium deoxycholate; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonate); LDAO, *N,N*-dimethyldodecylamine *N*-oxide.

Roseobacter denitrificans to the α -3 subclass of Proteobacteria.

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 (1:5, v/v) at 85 °C for 18 h and determined by combined gas liquid chromatography-mass spectrometry (GC-MS, Hewlett Packard HP 5988 A). The fatty acid methyl esters were separated on a fused silica capillary column DB 1 (length 15 m, i.d. 0.25 mm). Mass spectra were taken at 70 eV in the mass range 43–400 Da s⁻¹. For quantification, the fatty acid methyl esters were separated by gas-liquid chromatography (GLC; Varian Aerograph, model 3300) on an FFAP column [5% on Chromosorb, 80–100 mesh, length 1.8 m, i.d. 2 mm, with a temperature programme ranging from 150 to 200 °C (5 min initial temperature then with a ramp rate of 2 °C min⁻¹)]. Ester- and amide-linked fatty acids were liberated by hydroxylaminolysis (Snyder & Stephens, 1959). Neutral sugars, liberated by 0.1 M HCl at 100 °C for 48 h, were identified and quantified as their alditol acetate derivatives by GLC (Varian Aerograph, model 3700) on a fused silica capillary column DB 225 (length 30 m, i.d. 0.25 mm). For GC-MS analysis a Supelco SP 2380 capillary column was used. Mass spectra were taken at 70 eV in the mass range 43–400 Da s⁻¹. Amino sugars and amino acids, released by 4 M HCl at 100 °C for 18 h, were identified and quantified by combined amino acid/amino sugar analysis. The amino sugars were also identified as alditol acetates by GLC on a Poly A-103 column (length 1.8 m, i.d. 2 mm, 3% on Gaschrom Q, 100–200 mesh using a temperature programme from 200 to 260 °C, 5 min initial time followed by a ramp rate of 2 °C min⁻¹). Uronic acids were released by 0.5 M H₂SO₄ at 100 °C for 4 h and separated by high-voltage electrophoresis at 3 kV (80–120 mA and 4 °C for 3 h) in a solvent system (pyridine/formic acid/acetic acid/water, 1:1.5:10:90, by vol.) of pH 2.8. The electropherograms were stained with naphthoresorcinol according to Partridge (1948). 2-Keto-3-deoxyoctonate (KDO) was liberated by 1 M H₂SO₄ at 100 °C for 30 min and separated by high-voltage electrophoresis as described above except that the running time was reduced to 2 h. The electropherograms were stained with the periodate/thiobarbituric acid reagent according to Brade & Galanos (1983). Sialic acids were determined according to Yao *et al.* (1989); phosphorus was assayed according to Lowry *et al.* (1954).

Isolation of porin and determination of its sensitivity to EDTA. The cells were collected by centrifugation, washed with buffer (20 mM Tris/HCl, pH 8.0; 10 mM MgCl₂) and resuspended in the same buffer. DNase, RNase and phenylmethylsulfonyl fluoride were added and the cells disrupted by a Bühler homogenizer (type Vi2) for 25 min. The homogenate was centrifuged at 28 300 g for 1 h and washed once with the same buffer as above. The sediment (crude envelope fraction)

was extracted twice with 2% (v/v) Triton-X-100, 10 mM MgCl₂, 20 mM Tris/HCl, pH 8.0, at 40 °C and at 38 °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-dimethyldodecylamine 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 dialysed against 0.08% (w/v) LDAO, 20 mM NaCl, 2 mM NaN₃, 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 NaN₃, 20 mM Tris/HCl, pH 7.8. Subsequently the column was eluted with a linear salt gradient of 0.02–1 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 NaN₃, 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, I, 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 R_c-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 exclus-

Table 1. Chemical composition of the LPS, lipid A and polysaccharide fraction of *Roseobacter denitrificans*

Component	Content (nmol mg ⁻¹) in:		
	LPS	Lipid A	Polysaccharide fraction
Ribose	16.9	ND	15.1
Arabinose	5.5	ND	9.4
Mannose	24.8	ND	83.4
Glucose	39.2	ND	65.3
Glucosamine	238.5	385.0	—
Glucosamine 6-phosphate	39.8	11.2	—
Glycine	27.4	57.2	22.9
Alanine	0.7	—	1.0
Hexuronic acids	—	ND	ND
KDO	+	ND	ND
Phosphate	ND	1330	ND
3-Hydroxydecanoic acid	2.7	774.4	ND
3-Oxotetradecanoic acid	4.0	953.1	ND

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.

R. denitrificans
Rps. blastica
Rh. capsulatus
Rh. sphaeroides

D I T F D G F G R F G I
E I S L N G Y G R F G L
E F K L S G D A R M G V
E I S F S G Y A E M G G

Fig. 2. Alignment of the N-terminal ends of the porins from *Roseobacter denitrificans*, *Rhodopseudomonas blastica* (Kreusch *et al.*, 1994), *Rhodobacter capsulatus* (Schiltz *et al.*, 1991) and *Rhodobacter sphaeroides* (Woitzik *et al.*, 1990). Identical amino acids are boxed.

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*-Acetylneuraminic 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 derivatives. Uronic acids, examined by high-voltage electrophoresis and subsequent naphthoresorcinol staining, were not observed.

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 blastica* (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

ively ester-bound, and 3-oxotetradecanoic acid amide-bound, as determined by hydroxylaminolysis (Table 1). GC-MS analysis of the *N*-acetylated alditol acetates of the amino sugars revealed glucosamine but no diamino-glucose or any other amino sugar. The lipid A contained high amounts of phosphate. Small amounts of glycine and

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 & Mayer, 1988). *Rhodopseudomonas blastica*, 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_{GlcN} (Weckesser & Mayer, 1988). In other subgroups of the α -group there are species possessing non-phosphorylated lipid As, such as lipid A_{DAG} (Weckesser & Mayer, 1988) in *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* or lipid A_{GlcN} in *Rhodomicrobium vannielii* (Mayer & Weckesser, 1984). The LPS of *Roseobacter denitrificans* – and that of *Rhodopseudomonas blastica* – 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 blastica*, 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 cytochrome c_{551} and the B₈₇₀ 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 blastica* (Tegtmeyer *et al.*, 1985; Kawasaki *et al.*, 1993), renaming of *Roseobacter denitrificans* as *Rhodobacter denitrificans* would be the consequence.

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