Porphyrobacter mercurialis sp. nov., isolated from a stadium seat and emended description of the genus *Porphyrobacter*

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Abstract

Abstract

A novel, Gram-negative, non-spore-forming, pleomorphic yellow-orange bacterial strain was isolated from a stadium seat. Strain CoronadoT falls within the *Erythrobacteraceae* family and the genus *Porphyrobacter* based on 16S rRNA phylogenetic analysis. This strain has Q-10 as the predominant respiratory lipoquinone, as do other members of the family. The fatty acid profile of this strain is similar to other *Porphyrobacter*, however CoronadoT contains predominately C18:1ω7cis and C16:0, a high percentage of the latter not being observed in any other *Erythrobacteraceae*. This strain is catalase-positive and oxidase-negative, can grow from 4-28 °C, at NaCl concentrations 0.1-1.5%, and at pH 6.0-8.0. On the basis of phenotypic and phylogenetic data presented in this study, strain CoronadoT represents a novel species in the *Porphyrobacter* genus for which the name *Porphyrobacter mercurialis* sp. nov. is proposed; the type strain is CoronadoT (=DSMZ 29971, =LMG 28700).

INTRODUCTION

In this study, strain CoronadoT was isolated from a stadium seat at Niedermeyer Field, Coronado High School in Coronado, California, USA as part of a nationwide Citizen Science project (Project MERCCURI - www.spacemicrobes.org.) One goal of Project MERCCURI was to collect bacterial isolates to be used for an experiment aboard the International Space Station (ISS). A cotton swab was used to swab the surface of a stadium seat, then colonies were isolated via plating onto agar plates. The 16S rRNA gene was sequenced from this isolate and was at least 99% identical to rRNA genes from a few uncultured organisms. Uncultured isolates with high identity (=>99%) were found in samples from deep ocean sediment and the human skin microbiome. However, the highest identity to a cultured organism (*Porphyrobacter donghaensis*) [Error: Reference source not found] was only 95.5% (as determined by BLAST, [1]). Given the low identity to characterized species, a more detailed study of this isolate was undertaken.

A 2005 phylogenetic analysis of the *Alphaproteobacteria* class led to the creation of a new family, *Erythrobacteraceae*, to house the genera *Erythrobacter*, *Porphyrobacter* and *Erythromicrobium* [15]. These genera were later joined by *Altererythrobacter* [14] and *Croceicoccus* [24], the latter work also emended the description of the family. Members of the *Erythrobacteraceae* family are Gram-negative, aerobic bacteria that contain cartenoids, usually appearing pink, orange or yellow. They do not form spores, are chemo-organotrophic, and are most often associated with aquatic environments.

Phylogenetic and biochemical characteristics presented here show that this isolate is clearly distinct from other members within the *Erythrobacteraceae* family and is most closely related to the genus *Porphyrobacter*. However, a major taxonomic revision of this family is most likely required, as has been suggested by others (*e.g.* [18] [10]). Here we describe the genotypic, morphologic, and biochemical characteristics of strain CoronadoT, based on which we propose the name of *Porphyrobacter mercurialis* sp. nov.

METHODS

Cells were initially grown on plates containing either Reasoner's 2A agar (R2A), or lysogeny broth agar (LB). LB was made with 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter. A clear preference for growth on LB was

observed and so was used for all subsequent experiments. Salt tolerance was measured by growth in liquid media (25 °C) from 0% to 20% w/v NaCl. pH tolerance was measured by growth in liquid media (25 °C) from pH 3.4 to pH 8.0. Temperature preference was measured by growth in liquid culture across the range 4 °C to 30 °C. Growth in microgravity (OD600) was measured aboard the International Space Station (ISS).

Cell morphology, motility, and presence/absence of flagella were examined by light microscopy (Zeiss Axio Lab.A1) and transmission electron microscopy (TEM). Cell cultures in either exponential or stationary phase were prepared for TEM by the UC Davis Electron Microscopy lab as follows. 400 mesh copper grids with formvar/carbon support film (Ted Pella, Inc., Redding, Ca.) were placed on dental wax. A 10 µl drop of fixed or unfixed sample was placed onto the grid and left in a dust-free environment for 10 minutes. Then excess was wicked off with filter paper. A 10 µl drop of 1% PTA pH 5.8 (phosphotungstic acid) or 1% ammonium molybdate in double-distilled water was added to the grid and wicked off immediately. Grids were allowed to air-dry completely before viewing in a Philips CM120 (FEI/Philips Inc, Hillsborough, Or.) electron microscope at 80KV.

Oxidase activity was measured using a solution of tetramethyl-p-phenylenediamine and catalase activity was measured by the addition of hydrogen peroxide to plated cells. The hydrolysis of starch and casein were measured by standard plate methods (beef agar with soluble starch and iodine staining, and milk agar with a pancreatic digest of casein respectively). Carbon source oxidation was assayed using the Phenotypic MicroArray (TM) services offered by Biolog, Inc. using their standard procedures for gram-negative bacteria as follows. Colonies were grown on blood agar at room temperature and suspended in IF-0a inoculating fluid (Biolog) to a density of 42% transmittance. The cell suspension was diluted 1:6 in IF-0a plus 1x Dye H (Biolog) and a carbon source utilization MicroPlate (PM1; Biolog) was inoculated with 100 µl per well. The PM1 microplate was incubated at 23 °C and read by the OmniLog instrument every 15 minutes for 96 hours. Duplicate sets of OmniLog data were converted to average read value and a threshold of 78 was required in both replicates for a positive call.

Respiratory quinones, polar lips, and fatty acids

Cells were grown to post exponential phase (~72 hours) in 1 L of LB at 23 °C for large-scale biomass production, then centrifuged to pellet cells and lyopholized (VirTis Freezemobile). Analysis of respiratory quinones/polar lipids and fatty acids were carried out by the Identification Service, DSMZ, Braunschweig. Germany. Protocol details and references can be found at the following DSMZ pages; https://www.dsmz.de/services/services-microorganisms/identification/analysis-of-polar-lipids.html, https://www.dsmz.de/services/services-microorganisms/identification/analysis-of-respiratory-quinones.html, https://www.dsmz.de/services/services-microorganisms/identification/analysis-of-cellular-fatty-acids.html.

16S rDNA, genome sequencing, and phylogenetic analysis

Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega). A nearly full-length 16S rRNA gene sequence was amplified using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1391R (5'-GACGGGCGGTGTGTRCA-3') "universal" primers. Sanger sequencing was provided by the College of Biological Science UC-DNA Sequencing Facility (UC Davis). This DNA was also used for Illumina sequencing of the draft genome as described elsewhere (Coil and Eisen, in press). The genome sequence was annotated using the RAST server [2] [17].

The 1482bp 16S rDNA sequence was obtained from the genome assembly in RAST (GenBank: KP122961.1), and uploaded to the Ribosomal Database Project (RDP) [3]. Since the RDP database is incomplete with respect to the *Erythrobacteraceae* family, additional type strain sequences were obtained from NCBI to ensure that every member of the family with official standing in nomenclature (http://www.bacterio.net/) was present in the alignment downloaded from RDP. Because the taxon names exported with this alignment contained special characters that were not compatible with phylogenetic reconstruction software, a custom script was used to remove or replace those characters with underscores. A description of and link to this script can be found in [4]. The alignment was manually examined using MView (http://www.ebi.ac.uk/Tools/msa/mview/t), the secondary structure was generated using the RNAfold Web Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and visualized with Forna (http://nibiru.tbi.univie.ac.at/forna/). This alignment was used to generate phylogenetic trees using a variety of methods including maximum likelihood (RAxML, [21]), Bayesian (MrBayes, [19] [11]), neighbor-joining (MEGA6, [22], NINJA, http://nimbletwist.com/software/ninja/), and maximum parsimony (MEGA6). Dendroscope 3 [12] and FigTree (http://tree.bio.ed.ac.uk/software/figtree/) were used to view and edit the phylogenetic trees.

RESULTS AND DISCUSSION

Morphological, physiological, and biochemical characteristics

Cells were non-motile and not observed to form spores or possess flagella, though over 30 flagella or flagella-associated genes are present in the genome. In contrast, the genome for a non-motile close relative (*Porphyrobacter cryptus*, GenBank:16153) does not contain any flagellar genes so it is possible that strain CoronadoT is motile under

108 specific conditions. Unlike most members of the *Erythrobacteraceae family*, strain CoronadoT is oxidase-negative.
109 This strain is catalase-positive, and unable to hydrolze casein or starch.
110   Cells were oval or rod shaped and ranged in length from 1.2 μm to 2.2 μm with an average of 1.6 μm (Figure 1).
111 Cell width ranged from 0.6 μm to 1.0 μm with an average of 0.8 μm.
112   Growth was only observed under aerobic conditions, from 4 °C to 28 °C, with optimal growth around 25 °C. No
113 growth was observed under microaerophilic conditions (culture caps closed). Low levels of growth were observed at
114 pH 6.0 up to pH 8.0, maximum growth occurred around neutral pH. NaCl was required for growth, and the strain
115 could not grow at >1.5% NaCl, optimal growth was at 0.5% NaCl. No statistically significant difference in growth
116 was observed between earth and microgravity aboard the International Space Station (ISS).
117   Strain CoronadoT could oxidize the following as sole carbon sources: Glycyl-L-Glutamic Acid, L-Rhamnose, D-
118 Mannose, D-Trehalose, a-D-Glucose, L-Fucose, D-Galactose, Citric acid, D-Glucuronic acid, D-Galactonic acid, L-
119 Galactonic acid-γ-Lactone, Acetoacetic acid, Acetic acid, Pyruvic acid, and L-Malic acid.
120   The strain was unable to grow on N-Acetyl-D-Glucosamine, D-Saccharic Acid, Succinic Acid, L-Aspartic Acid,
121 L-Proline, D-Alanine, Dulcitol, D-Serine, D-Sorbitol, Glycerol, D-Gluconic Acid, D,L-α-Glycerol-Phosphate, L-
122 Lactic Acid, Formic Acid, D-Mannitol, L-Glutamic Acid, D-Glucose-6-Phosphate, D-Galactonic Acid-γ-Lactone,
123 D,L-Malic Acid, Tween 20, D-Fructose, Maltose, D-Melibiose, Thymidine, L-Asparagine, D-Aspartic Acid, D-
124 Glucosaminic Acid, 1,2-Propanediol, Tween 40, α-Keto-Glutaric Acid, α-Keto-Butyric Acid, α-Methyl-D-
125 Galactoside, α-D-Lactose, Lactulose, Sucrose, Uridine, L-Glutamine, m-Tartaric Acid, D-Glucose-1-Phosphate, D-
126 Fructose-6-Phosphate, Tween 80, α-Hydroxy Glutaric Acid-γ-Lactone, α-Hydroxy Butyric Acid, β-Methyl-D-
127 Glucoside, Adonitol, Maltotriose, 2-Deoxy Adenosine, Adenosine, Glycyl-L-Aspartic Acid, m-Inositol, D-Threonine,
128 Fumaric Acid, Bromo Succinic Acid, Propionic Acid, Mucic Acid, Glycolic Acid, Glyoxylic Acid, D-Cellobiose,
129 Inosine, Tricarballylic Acid, L-Serine, L-Threonine, L-Alanine, L-Alanyl-Glycine, Acetoacetic Acid, N-Acetyl-β-D-
130 Mannosamine, Mono Methyl Succinate, Methyl Pyruvate, D-Malic Acid, Glycyl-L-Proline, p-Hydroxy Phenyl
131 Acetic Acid, m-Hydroxy Phenyl Acetic Acid, Tyramine, D-Psicose, Glucuronamide, Phenylethyl-amine, or 2-
132 Aminoethane.

133 **Phylogeny and Genome analysis**
134   Phylogenetic analysis was performed using the full length (1482 bp) 16S rDNA sequence from the genome
135 assembly, not the shorter (1350 bp) version from Sanger sequencing. The CoronadoT 16S rDNA sequence showed
136 less than 95.5% identity to other *Porphyrobacter* species and identity is even lower to other genera in the family.
137 Given the low 16S rDNA identity to other members of the family, we did not perform DNA-DNA hybridization as
138 this would have been uninformative [20] [23] [16].
139   Phylogenetic trees built by varying the alignment and tree-building algorithms, number of taxa included, and
140 choice of outgroup demonstrated both that the current taxonomy of the family is in need of revision (as has been
141 suggested by others, *e.g.,* [18] and [10]) and that the placement of CoronadoT within the family is not stable.
142 Because the Bayesian and Maximum Likelihood methods show a very similar topology, and are considered the most
143 accurate methods for phylogenetic analysis (e.g. [25], [13], [6]), we have shown those trees in Figure 2 and Figure 3.
144 Both of these trees place CoronadoT within the *Porphyrobacter* clade, though in the Bayesian tree there is a
145 polytomy at the base of this clade. We note, as also shown recently by [10], that this clade is always polyphyletic
146 with respect to *Erythromicrobium ramosum* and often to *Erythrobacter litoralis*. In addition, CoronadoT is found on
147 a long branch due to several changes that are unique to this strain, relative to the rest of the family. These changes are
148 identical in both the assembly and the Sanger sequence and are all compatible with the secondary structure model of
149 16S (*e.g.,* changes in a stem nucleotide pair with the appropriate base). Based on this analysis, we chose to compare
150 CoronadoT to the five other *Porphyrobacter* species listed in Table 1.
151   Analysis of the draft genome of strain CoronadoT was used to complement the physical characterizations typical
152 of the family *Erythrobacteraceae* and the genus *Porphyrobacter*. For example, CoronadoT does not contain any of
153 the numerous genes involved in bacteriochlorophyll biosynthesis, rendering protein extraction/spectrophotometry
154 unnecessary. Conversely, while no flagella were observed by TEM, this strain appears to possess the required genes
155 making it likely that the flagella were lost in sample preparation or that their expression is condition-dependent.

156 **Polar lipid, respitory lipoquinone, and fatty acid methyl esters**
157   The major cellular fatty acids of strain CoronadoT are C18:1ω7cis (56.6%) and C16:0 (20.3%). Other fatty acids
158 found in significant amounts (>1%) are 2-OH-C14:0 (4.8%), C16:1ω5cis (1.1%), C16:1ω7cis (9.8%), C17:1ω6cis
159 (2%), C18:1ω5cis (1.1%), and C18:0 (1.2%). The fatty acid profile of strain Coronado(T) fits generally within the
160 ranges described for members of the most closely related genera (*Erythrobacter*, *Porphyrobacter* and
161 *Erythromicrobium*, comparison data from [9]). The two exceptions to this are a slightly higher level of C14:0 than
162 average and a much higher level of C16:0 than average. However, it is difficult to compare across studies since
163 variation in growth conditions can significantly influence the fatty acid profile.
164   The major respiratory quinone is ubiquinone 10 (92%), as it is for all members of the *Erythrobacteraceae* family.
165 The predominant polar lipid is phosphatidylglycerol, with significant amounts of sphingoglycolipid and

166 phosphatidylethanolamine. Smaller amounts of diphosphatidylglycerol, phosphatidylcholine, and two unidentified
167 phospholipids were also observed (Figure 4).
168   **Conclusions**
169   Strain CoronadoT clearly falls within the *Erythrobacteraceae* family, based on both phylogenetic analysis and
170 physical characteristics (notably fatty acid profile, carotenoid production, major respiratory quinone, and GC
171 content). Using Bayesian and Maximum Likelihood phylogenetic reconstruction, the strain falls within a well-
172 supported (but polyphyletic) *Porphyrobacter* clade. In addition, CoronadoT shares a number of characteristics with
173 *Porphyrobacter*, including the fatty acid profile, polar lipid composition, catalase activity, etc. The largest differences
174 are the tolerance for growth at lower temperatures, elevated C16:0, and the lack of bacteriochlorophyll *a* (for the
175 latter of which we have proposed emending the genus description). These characteristics, in combination with the
176 phylogenetic analysis, lead us to propose that CoronadoT be classified as *Porphyrobacter mercurialis* sp. nov. We are
177 aware that a future in depth polyphasic comparative study and/or a genome sequencing based taxonomic revision of
178 the entire family may merit a distinct genus for this this strain.

0.1 Emended description of the genus *Porphyrobacter* Fuerst et al 1993

181 The description is identical to that given by [5] with the following amendments. Most species synthesize BChl *a* on
182 low-nutrient media under aerobic and semiaerobic conditions. DNA base composition is 63.8 to 67.3 G+C.

0.2 Description of *Porphyrobacter mercurialis* sp. nov.

184 *Porphyrobacter mercurialis* (mer.cur.ia.al'is L. adj. *mercurialis*, temperamental in reference to difficulties in
185 establishing consistent growth requirements during characterization).
186   Gram-negative, non-spore forming pleomorphic bacteria. Strictly aerobic and chemoheterotrophic. Contains
187 carotenoids, but not bacteriochlorophyll *a*. The major respiratory quinone is ubiquinone 10, the dominant
188 phospholipds are phosphatidylglycerol, sphingoglycolipid, and phophatidylthanolamine. Colonies on LB are round,
189 glossy, and less than 1 mm in diameter. Coloration on agar and in dense liquid culture is dark yellowish orange,
190 appears lighter yellow in liquid culture during exponential phase. Morphology is non-motile pleomorphic ovals or
191 short rods (average 1.6 μm length), often with pointed ends. Growth in liquid culture from 4-28 °C, optimum growth
192 at 25 °C. Requires salt for growth, optimal growth at 0.5% NaCl, cannot grow at 3% NaCl. Tolerates up to pH 8.0,
193 optimum around neutral pH. Oxidase-negative and catalase-positive, does not hydrolyze casein or starch. Does not
194 grow on R2A agar. The primary fatty acids are C18:1ω7cis and C16:0, with a high percentage of the latter relative to
195 other species in the *Erythrobacteraceae* family. Can oxidize the following as sole carbon sources: Glycyl-L-
196 Glutamic Acid, L-Rhamnose, D-Mannose, D-Trehalose, a-D-Glucose, L-Fucose, D-Galactose, Citric acid, D-
197 Glucuronic acid, D-Galactonic acid, L-Galactonic acid-γ-Lactone, Acetoacetic acid, Acetic acid, Pyruvic acid, and L-
198 Malic acid.
199   The type strain CoronadoT (=DSMZ 29971, =LMG 28700) was isolated from a stadium seat in Coronado,
200 California, USA. The GC content of the type strain is 67.3%, as determined by genome sequencing. The genome size
201 is approximately 3.5 MB.
202   **Acknowledgements**
203   The authors would like to thank the Coronado Pop Warner Islanders for initial collection of the sample and
204 participation in Project MERCCURI, as well as Kris Tracy who assisted in the etymology of the proposed species
205 name.

0.3 Table 1

207 Caption for Table 1 (Placed in document so that the appropriate references appear in the Bibliography, please remove
208 in actual paper)
209   Phenotypic comparison of CoronadoT and other members of the *Porphyrobacter* genus. Positive=+, negative=-,
210 No data available=ND. Data from this study and [8], [18], [7], [27], [5], [26].

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Figure 1: Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) of exponential phase culture of Coronado[T] in lysogeny broth (LB), grown at 23°C. Cells were negatively stained with Ammonium Molybdate.

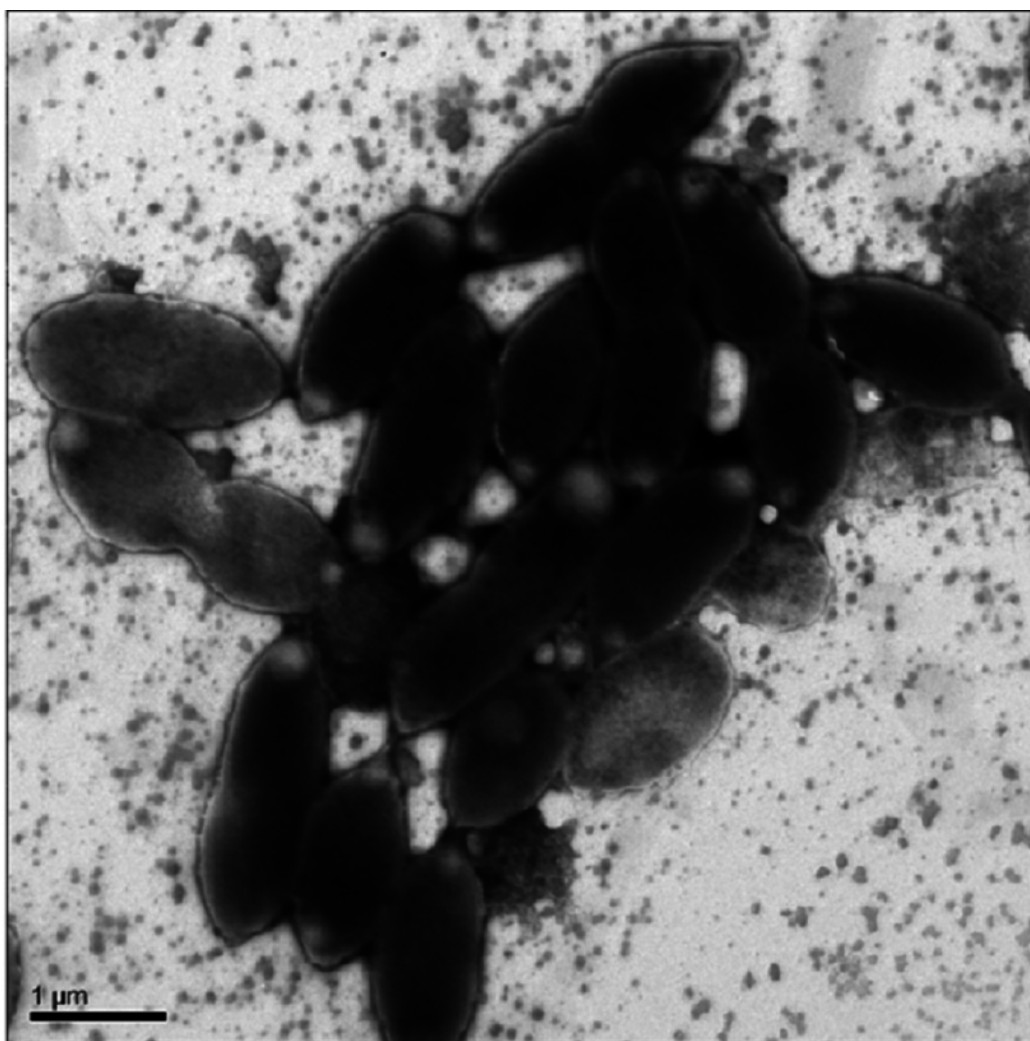



Figure 2: Maximum Likelihood tree

Figure 2: Maximum Likelihood tree based on the 16S rRNA gene sequence of Coronado^T and all type strains from the *Erythrobacteraceae* family. Tree was inferred from an Infernal-based alignment created in RDP using RAxML with the gamma model of rate heterogeneity. Numbers at the nodes (only values >70 are shown) represent support values for 1000 bootstrap replicates. The tree was rooted to *Novosphingobium indicum* as an outgroup since this species was shown to be one of the closest relatives to the *Erythrobacteraceae* family in a tree of all *Alphaproteobacteria*.

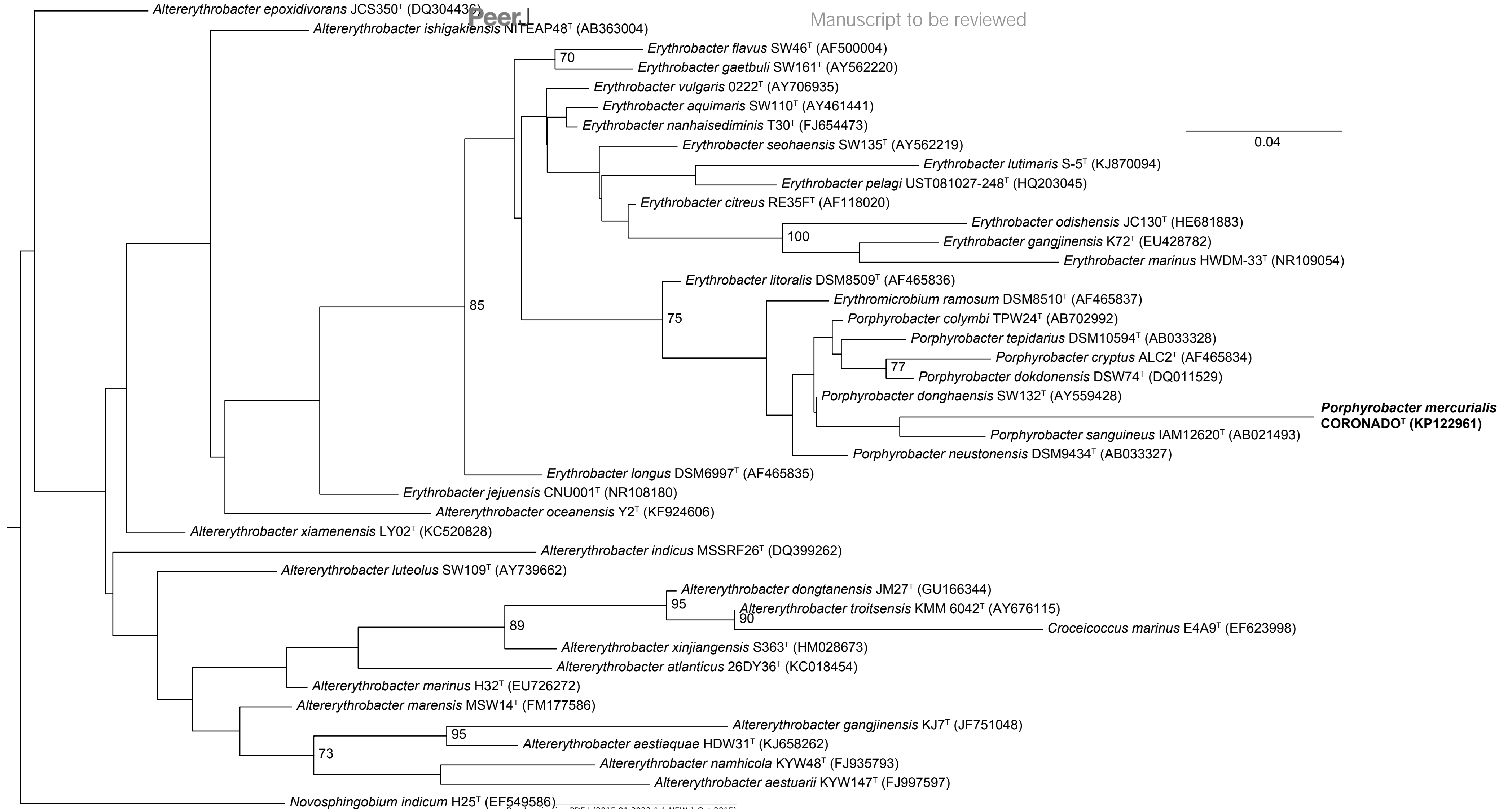



Figure 3: Bayesian tree

Figure 3: Bayesian tree based on the same sequence alignment described in Figure 2 (Infernal-based RDP alignment of 16S rRNA gene sequence of Coronado[T] and all type strains from the *Erythrobacteraceae* family). Tree was inferred using MrBayes with the General Time Reversible (GTR) substitution model. Numbers at nodes (only values >70 are shown) represent posterior probability values from 1,000,000 iterations of the tree, with a 25% relative burnin. The tree was rooted to *Novosphingobium indicum* as an outgroup since this species was shown to be one of the closest relatives to the *Erythrobacteraceae* family in a tree of all *Alphaproteobacteria*.

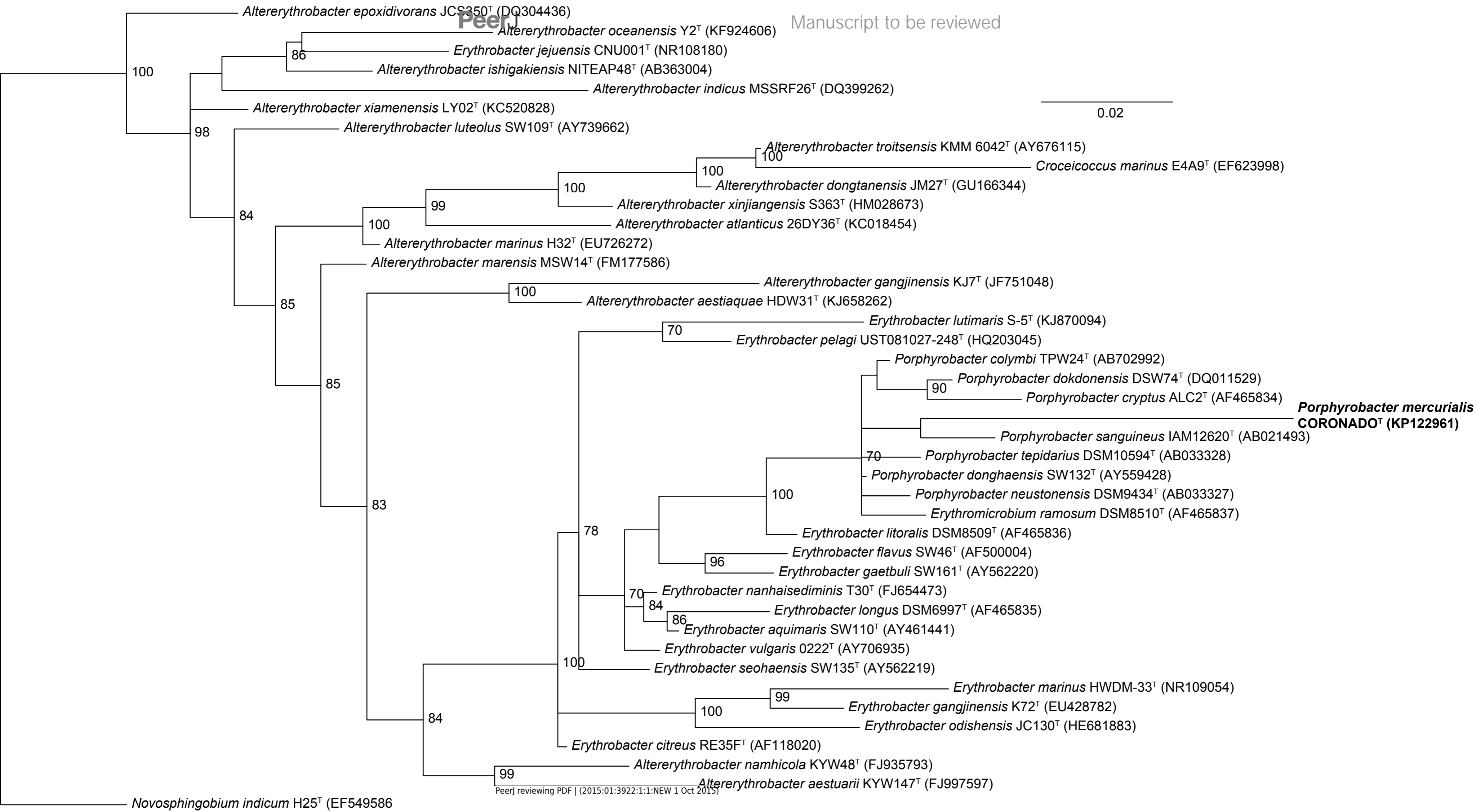
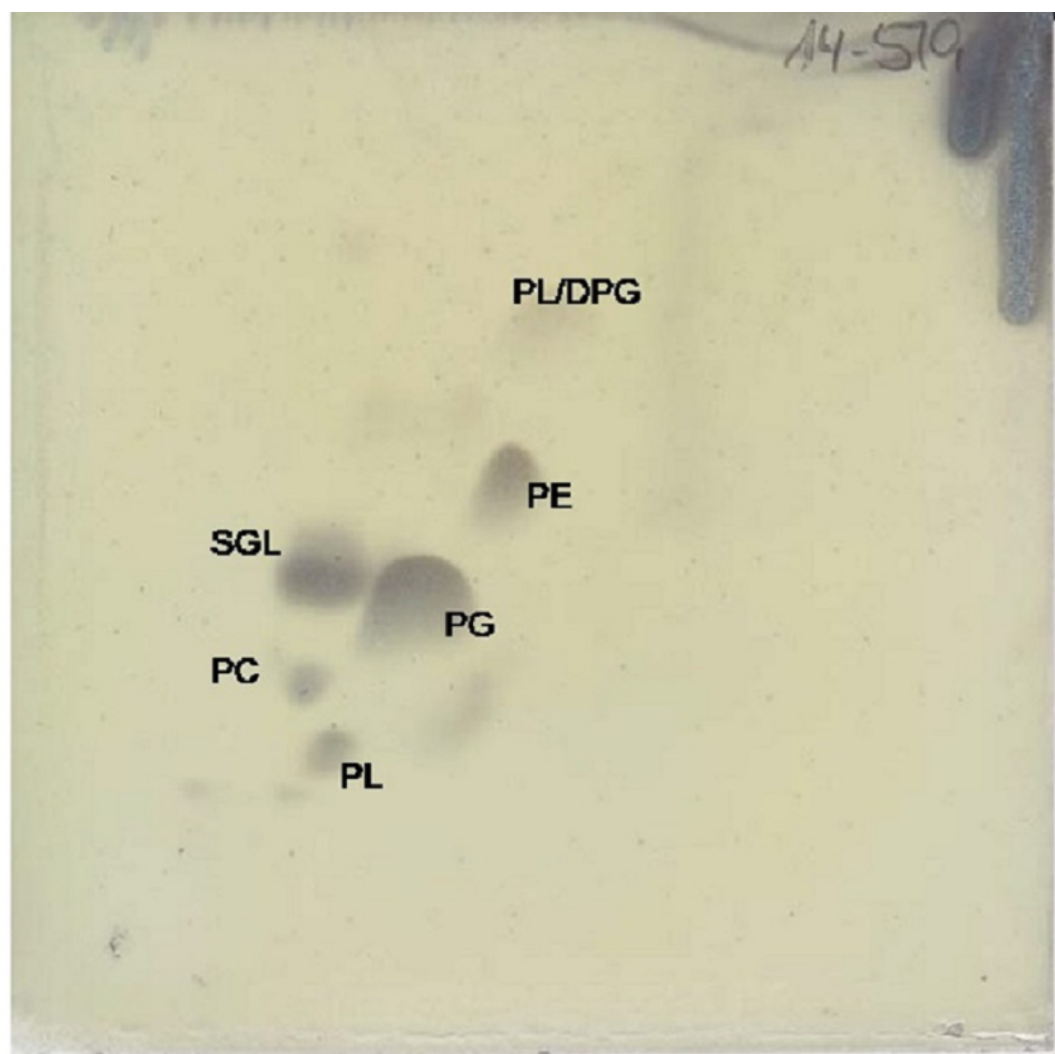



Figure 4: Two dimensional silica gel thin layer chromatography results for polar lipids, isolated following standard protocols at the DSMZ (see Methods).

PL=phospholipid, PG=phosphatidylglycerol, PC=phosphatidylcholine,

PE=phosphatidylethanolamine, DPG=diphosphatidylglycerol, SGL=Sphingoglycolipid.

Phenotypic comparison of Coronado[T] and other members of the Porphyrobacter genus

*C, Cocci; O, oval; R, rod. †Y, Yellow; O, orange; R, red. Positive=+, negative=-, No data available=ND. Data from this study and (Hiraishi 2002a) , (Rainey 2003) , (Hanada 1997) , (Yoon 2004) , (Fuerst 1993) , (Yoon 2006) .

Characteristic	strain CoronadoT	*P. sanguineus*	*P. tepidarius*	*P. donghaensis*	*P. cryptus*	*P. neustonensis*
Cell shape	O/R	R	O/R	C/O/R	R	C/O/R
Color of colony	YO	OR	O	OR	OR	OR
Motility	-	+	-	-	+	+
Presence of BChl a	-	+	+	+	+	+
Growth in NaCl (%):						
Range	.1-1.5	ND	0.0-1.3	ND	ND	ND
Optimum	.5	1	ND	2	ND	ND
Growth pH:						
Range	6.0-8.0	ND	ND	ND	6.0-9.0	ND
Optimum	7.0	7.0-7.5	ND	7.0-8.0	7.5-8.0	ND
Growth temperature (°C)						
Range	4-28	20-37	ND	10-45	ND	10-37
Optimum	25	30	40-48	30-37	50	ND
Catalase	+	+	ND	+	+	+
Oxidase	-	+	-	+	+	-
Major cellular fatty acids	$C_{18:1}\omega7cis$, C_{16}	$C_{18:1}\omega7cis$	ND	$C_{18:1}\omega7cis$, $C_{17:1}\omega6cis$	$C_{18:1}\omega7cis$	n18:1
DNA G + C content	67.3	63.8-64 (mol%)	65 (mol%)	65.9-66.8 (mol%)	66.2 (mol%)	65.7-66.4 (mol%)

2   *C, Cocci; O, oval; R, rod.

3   †Y, Yellow; O, orange; R, red

4   Phenotypic comparison of Coronado[T] and other members of the *Porphyrobacter* genus. Positive=+,
5   negative=-, No data available=ND. Data from this study and (Hiraishi 2002a), (Rainey 2003), (Hanada
6   1997), (Yoon 2004), (Fuerst 1993), (Yoon 2006).