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ABSTRACT

A novel, Gram-negative, non-spore-forming, pleomorphic yellow-orange bacterial strain was isolated from a stadium seat. Strain Coronado(T) falls within the *Erythrobacteraceae* family based on 16S rRNA phylogenetic analysis, but is both phylogenetically and physiologically distinct from existing genera in the family. A phylogenetic tree inferred from 16S rRNA gene sequences shows a highly supported clade containing Coronado(T), *Porphyrobacter*, *Erythromicrobium*, and *Erythrobacter*. While 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 distinct. Coronado(T) contains predominatly 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, the latter of which is unusual for the other genera present in the same clade. Coronado(T) 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 Coronado(T) represents a novel species in a new genus in the family *Erythrobacteraceae* for which the name *Kirrobacter mercurialis* gen. nov., sp. nov. is proposed; the type strain is Coronado(T) (=DSMZ 29971, =LMG 28700).

INTRODUCTION

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* [7]. These genera were later joined by *Altererythrobacter* [6] and *Croceicoccus* [13], 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.

In this study, strain Coronado(T) 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. Almost full length (primers 27F and 1391R) 16S rRNA sequencing showed 99% identity to a few uncultured sequences at NCBI, but no more than 95% identity to the nearest cultured representative (*Porphyrobacter donghaensis*)[14]. Uncultured isolates with high identity (=>99%) were found in studies of deep ocean sediment and the human skin microbiome.

Phylogenetic and biochemical characteristics show that this isolate belongs to a new genus and species within the *Erythrobacteraceae* family. However, phylogenetic analysis of the entire family would suggest that a major taxonomic revision of the entire group is required, as has been suggested by others (*e.g.* [10]) Here we describe the genotypic, morphologic, and biochemical

54 characteristics of strain Coronado(T), which represents a novel genus and species. We propose
55 the name of *Kirrobacter mercurialis* gen. nov., sp. nov.
56
57 **METHODS**
58 Cells were initially grown on plates containing either Reasoner's 2A agar (R2A), or lysogeny
59 broth agar (LB). LB was made with 10g tryptone, 10g NaCl, and 5g yeast extract per liter. A
60 clear preference for LB was observed and so was used for all subsequent experiments. Salt
61 tolerance was measured in liquid media (25°C) from 0% to 20% w/v NaCl. pH tolerance was
62 measured in liquid media (25°C) from pH 3.4 to pH 8.0. Temperature preference was measured
63 in liquid culture across the range 4°C to 30°C. Growth in microgravity was measured aboard the
64 International Space Station (ISS).
65 Cell morphology, motility, and presence/absence of flagella were examined by light
66 microscopy (Zeiss Axio Lab.A1) and transmission electron microscopy (TEM). Cell cultures in
67 either exponential or stationary phase were prepared for TEM by the UC Davis Electron
68 Microscopy lab as follows. 400 mesh copper grids with formvar/carbon support film (Ted Pella,
69 Inc., Redding, Ca.) were placed on dental wax. A 10µl drop of fixed or unfixed sample was
70 placed onto the grid and left in a dust-free environment for 10 min. Then excess was wicked off
71 with filter paper. A 10µl drop of 1% PTA pH 5.8 (phosphotungstic acid) or 1% ammonium
72 molybdate in DDH2O was added to the grid and wicked off immediately. Grids were allowed to
73 air-dry completely before viewing in a Philips CM120 (FEI/Philips Inc, Hillsborough, Or.)
74 electron microscope at 80KV.
75 Catalase and oxidase activity, as well as the hydrolysis of starch and casein, were assayed by
76 standard methods. Carbon source utilization was assayed using the Phenotypic MicroArray(TM)

services offered by Biolog, Inc. following standard procedures for gram-negative bacteria. 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 100µl 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 in 1L of LB at 23°C for large-scale biomass production, then centrifuged and lyopholized. Analysis of respiratory quinones/polar lipids and fatty acids were carried out by the Identification Service, DSMZ, Braunschweig. Germany. Details are available on their website (http://www.dsmz.de/services/services-microorganisms/identification.html).

16S rDNA and Genome Sequencing

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, submitted). The genome sequence was annotated using the RAST server [1] [8].

A phylogentic tree was inferred using all type strains from the *Erythrobacteraceae* family. The 1482bp 16S rDNA sequence was obtained from the genome assembly in RAST, and uploaded to the Ribosomal Database Project (RDP) [2]. RDP was used to build an alignment

including every type strain within the *Erythrobacteraceae* family, including strain Coronado(T). 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 [3]. FastTree [9] was used with default settings to build an unrooted phylogeny. Dendroscope 3 [5] was used to view and edit the phylogenetic tree in order to 1) root the tree with a clade that is sister to a well-supported clade containing strain Coronado(T), 2) prune the tree to remove taxa outside the clade containg the outgroup, and 3) "clean up" the tree.

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 (*P. cryptus*) does not contain any flagellar genes so it is possible that strain Coronado(T) is motile under specific conditions. Unlike most members of the *Erythrobacteraceae family*, strain Coronado(T) is oxidase-negative. This strain is catalase-positive, and unable to hydrolze casein or starch.

Cells were oval or rod shaped and ranged in length from 1.2um to 2.2um with an average of 1.6um (Figure 1). Cell width ranged from 0.6um to 1um with an average of 0.8um.

Growth was only observed under aerobic conditions, from 4°C to 28°C, with optimal growth around 25°C. Low levels of growth were observed at pH 6.0 up to pH 8.0, maximum growth occurred around neutral pH. Salt was required for growth, and the strain could not grow at

>1.5% NaCl, optimal growth was at 0.5% NaCl. No statistically significant difference in growth was observed between earth and microgravity aboard the International Space Station (ISS).

Strain Coronado(T) could utilize the following as sole carbon sources: Glycyl-L-Glutamic Acid, L-Rhamnose, D-Mannose, D-Trehalose, a-D-Glucose, L-Fucose, D-Galactose, Citric acid, D-Glucuronic acid, D-Galactonic acid, L-Galactonic acid-γ-Lactone, Acetoacetic acid, Acetic acid, Pyruvic acid, and L-Malic acid.

The strain was unable to grow on N-Acetyl-D-Glucosamine, D-Saccharic Acid, Succinic Acid, L-Aspartic Acid, L-Proline, D-Alanine, Dulcitol, D-Serine, D-Sorbitol, Glycerol, D-Gluconic Acid, D,L-α-Glycerol-Phosphate, L-Lactic Acid, Formic Acid, D-Mannitol, L-Glutamic Acid, D-Glucose-6-Phosphate, D-Galactonic Acid-γ-Lactone, D,L-Malic Acid, Tween 20, D-Fructose, Maltose, D-Melibiose, Thymidine, L-Asparagine, D-Aspartic Acid, D-Glucosaminic Acid, 1,2-Propanediol, Tween 40, α-Keto-Glutaric Acid, α-Keto-Butyric Acid, α-Methyl-D-Galactoside, α-D-Lactose, Lactulose, Sucrose, Uridine, L-Glutamine, m-Tartaric Acid, D-Glucose-1-Phosphate, D-Fructose-6-Phosphate, Tween 80, α-Hydroxy Glutaric Acid-γ-Lactone, α-Hydroxy Butyric Acid, β-Methyl-D-Glucoside, Adonitol, Maltotriose, 2-Deoxy Adenosine, Adenosine, Glycyl-L-Aspartic Acid, m-Inositol, D-Threonine, Fumaric Acid, Bromo Succinic Acid, Propionic Acid, Mucic Acid, Glycolic Acid, Glyoxylic Acid, D-Cellobiose, Inosine, Tricarballylic Acid, L-Serine, L-Threonine, L-Alanine, L-Alanyl-Glycine, Acetoacetic Acid, N-Acetyl-β-D-Mannosamine, Mono Methyl Succinate, Methyl Pyruvate, D-Malic Acid, Glycyl-L-Proline, p-Hydroxy Phenyl Acetic Acid, m-Hydroxy Phenyl Acetic Acid, Tyramine, D-Psicose, Glucuronamide, Phenylethyl-amine, or 2-Aminoethane.

Phylogeny and Genome analysis

Phylogenetic analysis was performed using the full length (1482bp) 16S rDNA sequence from the genome assembly, not the shorter (1350bp) version from Sanger sequencing. The Coronado(T) 16S rDNA sequence showed only 95% identity to the phylogenetically closest relative, *Porphyrobacter sanquineus*, and identity was even lower throughout the rest of the tree. Given the low 16S rDNA identity to other members of the family, we did not perform DNA-DNA hybridization as this would have been uninformative [11] [12].

As discussed above, a large-scale taxonomic revision of the family is most likely in order given the lack of monophyly observed for most genera in the tree (Figure 2). For example, Coronado(T) is the basal member of a clade containing mostly *Porphyrobacter* that has strong bootstrap support, but is polyphyletic with respect to both *Erythromicrobium* and *Erythrobacter*. And, this clade falls within a well-supported polyphyletic clade of *Erythrobacter*. Based on this tree, we chose to compare Coronado(T) to the three genera in Table 1.

Analysis of the draft genome of strain Coronado(T) was used to complement the physical characterizations typical of the family *Erythrobacteraceae*. For example, Coronado(T) does not contain any of the numerous genes involved in chlorophyll biosynthesis, rendering protein extraction/spectrophotometry unnecessary. Conversly, while no flagella were observed by TEM, this strain appears to possess the required genes making it likely that the flagella were lost in sample preparation or that their expression is condition-dependent.

Polar lipid, respitory lipoquinone, and fatty acid methyl esters

The major cellular fatty acids of strain Coronado(T) are C18:1ω7cis (56.6%) and C16:0 (20.3%). Other fatty acids found in significant amounts (>1%) are 2-OH-C14:0 (4.8%), C16:1ω5cis (1.1%), C16:1ω7cis (9.8%), C17:1ω6cis (2%), C18:1ω5cis (1.1%), and C18:0 (1.2%). The fatty acid profile of strain Coronado(T) fits generally within the ranges described for

members of the most closely related genera (*Erythrobacter*, *Porphyrobacter* and *Erythromicrobium*, comparison data from [4]). The two exceptions to this are a slightly higher level of C14:0 than average and a much higher level of C16:0 than average.

The major respiratory quinone is ubiquinone 10 (92%), as it is for all members of the *Erythrobacteraceae* family. The predominant polar lipid is phosphatidylglycerol, with significant amounts of sphingoglycolipid and phosphatidylethanolamine. Smaller amounts of diphosphatidylglycerol, phosphatidylcholine, and two unidentified phospholipids were also observed (Figure 3).

Description of *Kirrobacter* gen. nov.

Kirrobacter (Kir.ro.bac'ter. Gr. adj. *kirros*, orange-tawny; Gr. adj. *bacter*, rod; N.L. n. *Kirrobacter*, orange-tawny colored rod)

Gram-negative, non-spore forming pleomorphic bacteria. Strictly aerobic and chemoheterotrophic. Contains carotenoids, but not bacteriochlorophyll *a*. The major respiratory quinone is ubiquinone 10, the dominant phospholipds are phosphatidylglycerol, sphingoglycolipid, and phophatidylthanolamine. The genus is located in a sub-clade within the *Erythrobacteraceae* family. The type species is *Kirrobacter mercurialis*.

Description of *Kirrobacter mercurialis* sp. nov.

Kirrobacter mercurialis (mer.cur.ia.al'is L. adj. *mercurialis*, temperamental).

In addition to the characteristics of the genus, colonies on LB are round, glossy, and less than 1 mm in diameter. Coloration on agar and in dense liquid culture is dark yellowish orange, appears lighter yellow in liquid culture during exponential phase. Morphology is non-motile pleomorphic ovals or short rods (average 1.6um length), often with pointed ends. Growth in

liquid culture from 4-28°C, optimum growth at 25°C. Requires salt for growth, optimal growth at 0.5% NaCl, cannot grow at 3% NaCl. Tolerates up to pH 8.0, optimum around neutral pH. Oxidase-negative and catalase-positive. Does not grow on R2A agar. The primary fatty acids are C18:1ω7cis and C16:0, with a high percentage of the latter relative to other genera in the family. Can utilize the following as sole carbon sources: Glycyl-L-Glutamic Acid, L-Rhamnose, D-Mannose, D-Trehalose, a-D-Glucose, L-Fucose, D-Galactose, Citric acid, D-Glucuronic acid, D-Galactonic acid, L-Galactonic acid-γ-Lactone, Acetoacetic acid, Acetic acid, Pyruvic acid, and L-Malic acid.

The type strain Coronado(T) (=DSMZ 29971, =LMG 28700) was isolated from a stadium seat in Coronado, CA. The GC content of the type strain is 67.3%, as determined by genome sequencing. The genome size is approximately 3.5MB.

Acknowledgements

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209

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1

Figure 1: Transmission Electron Microscopy (TEM)

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

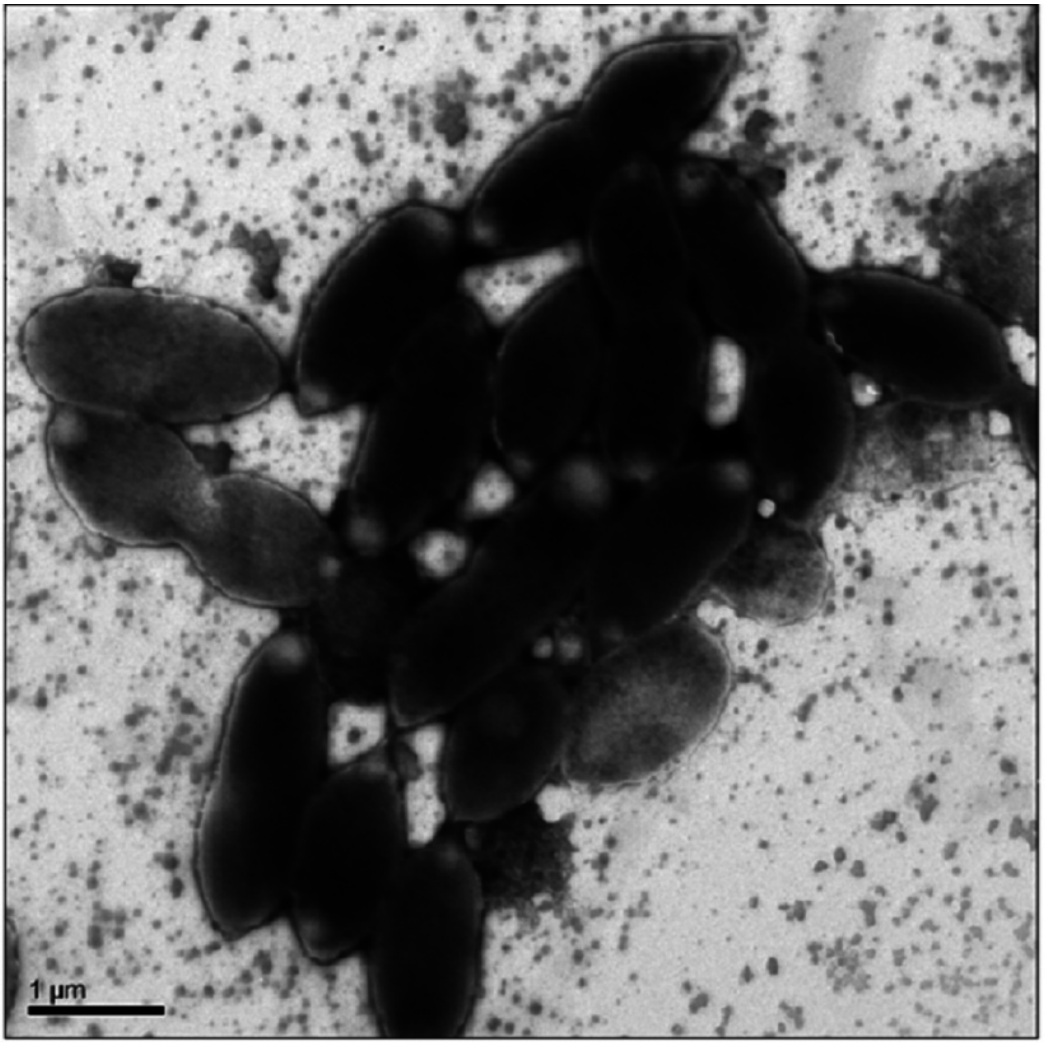
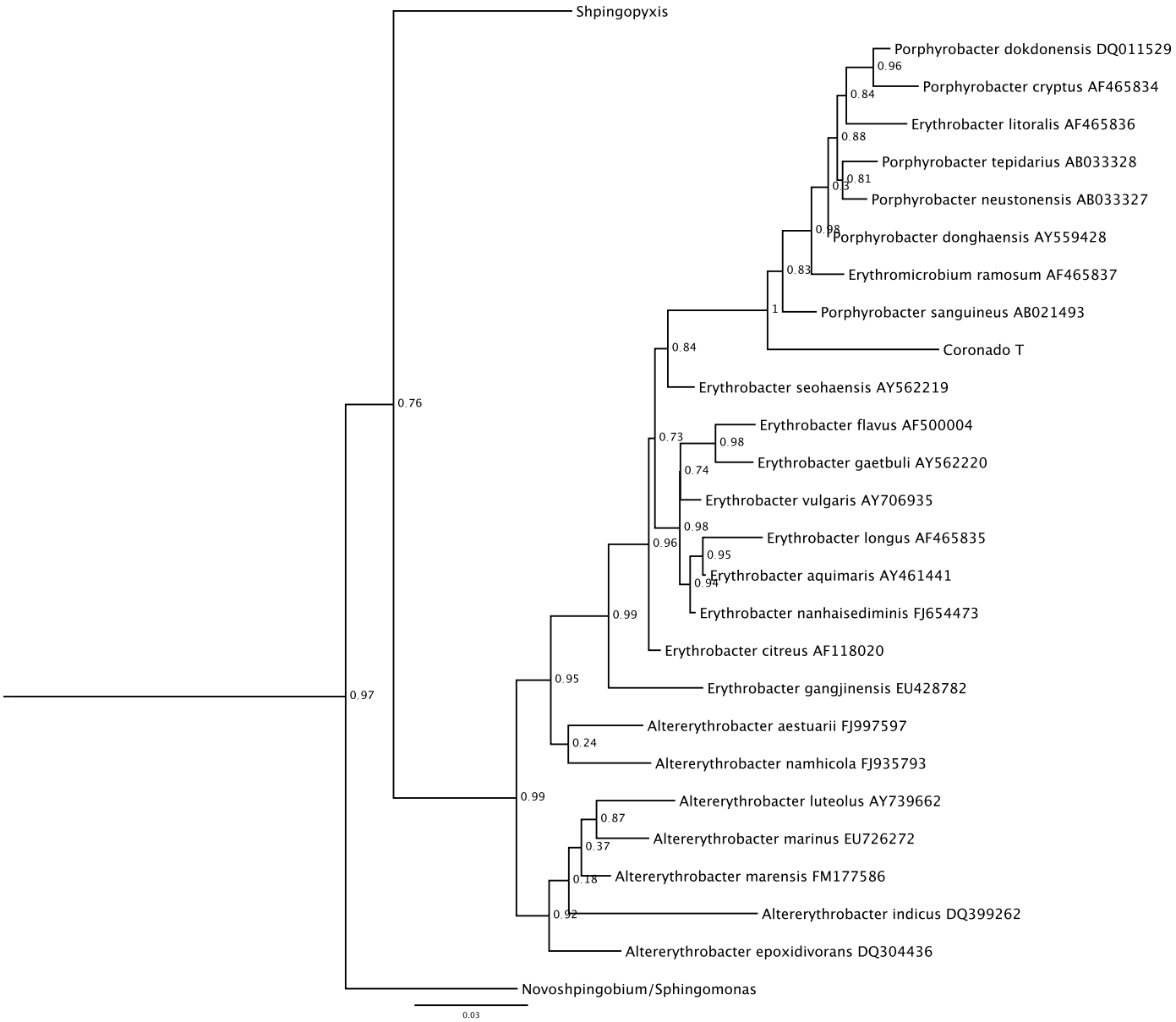


Figure 2(on next page)

Figure 2: Phylogenetic tree derived from 16S rRNA gene sequences of Coronado(T) and representative type strains

An approximate maximum likelihood phylogenetic tree was inferred with FastTree, based on 1482bp of the 16S rRNA gene sequences obtained from representatives of the *Erythrobacteraceae* family. Above the nodes are Shimodaira-Hasegawa-like local support values.


Shpingopyxis
Porphyrobacter dokdonensis DQ011529
Porphyrobacter cryptus AF465834
Erythrobacter litoralis AF465836
Porphyrobacter tepidarius AB033328
Porphyrobacter neustonensis AB033327
Porphyrobacter donghaensis AY559428
Erythromicrobium ramosum AF465837
Porphyrobacter sanguineus AB021493
Coronado T
Erythrobacter seohaensis AY562219
Erythrobacter flavus AF500004
Erythrobacter gaetbuli AY562220
Erythrobacter vulgaris AY706935
Erythrobacter longus AF465835
Erythrobacter aquimaris AY461441
Erythrobacter nanhaisediminis FJ654473
Erythrobacter citreus AF118020
Erythrobacter gangjinensis EU428782
Altererythrobacter aestuarii FJ997597
Altererythrobacter namhicola FJ935793
Altererythrobacter luteolus AY739662
Altererythrobacter marinus EU726272
Altererythrobacter marensis FM177586
Altererythrobacter indicus DQ399262
Altererythrobacter epoxidivorans DQ304436
Novoshpingobium/Sphingomonas
0.03

3

Figure 3: Two dimensional silica gel thin layer chromatography (TLC) results for polar lipids.

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

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

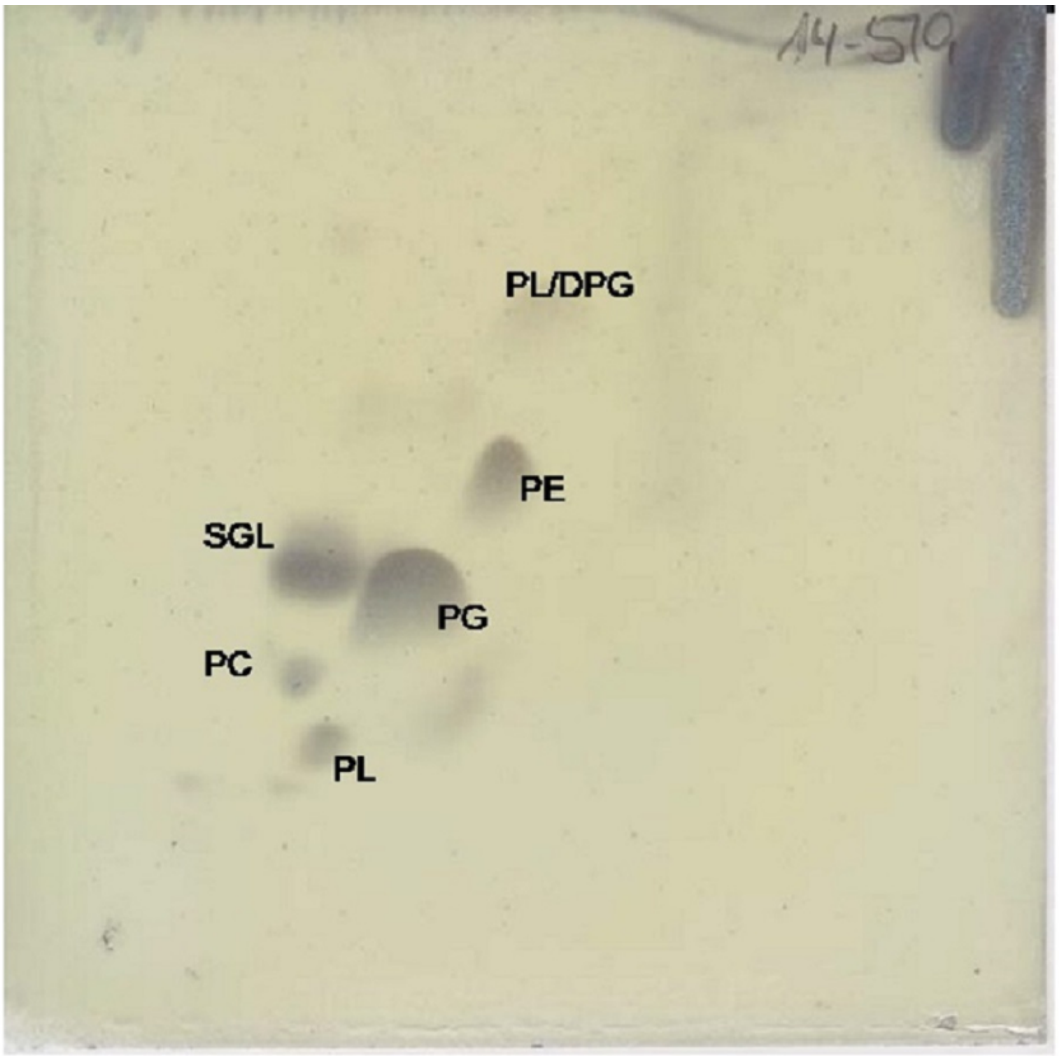


Table 1(on next page)

Table 1: Phenotypic comparison of Coronado(T) and other members of the same clade within the *Erythrobacteraceae* family.

Data from Xu 2009 and references therein. Data refer to all species within the listed genera. Positive=+, negative=-, positive or negative reported for more than half of species but no data for others=(+) and (-) No data available=NA, variable=V, variable but more than half having positive or negative results=V(+) and V(-).

1

Characteristic	strain Coronado(T)	*Erthrobacter*	*Erythromicrobium*	*Porphyrobacter*
Cell shape	O/R	R	R	C/O/R
Color of colony	YO	Y/O/R	O	O/R
Motility	-	V(-)	+	V
Growth in NaCl (%):				
Range	.5-1.5	.1-9.0	NA	0-7
Optimum	.5	.2-5.0	NA	1-2
Growth pH:				
Range	6.0-8.0	6.0-10.0	NA	6.0-9.0
Optimum	7.0	6.5-8.5	7.0-8.5	6.5-8.5
Growth temperature (°C)				
Range	4-28	10-43	NA	10-55
Optimum	25	25-37	25-30	30-50
Catalase	+	+	+	(+)
Oxidase	-	+	+	V(+)
Major cellular fatty acids	$C_{18:1}\omega7cis$, C_{16}	$C_{18:1}\omega7cis$, $C_{17:1}\omega6cis$	$C_{17:1}\omega6cis$, $_{18:1}\omega7cis$	$C_{18:1}\omega7cis$, $C_{17:1}\omega6cis$
DNA G + C content	67.3	60-67	62.5-68.5	63.8-66.8

2

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

†Y, Yellow; O, orange; R, red.

3

4 Table1: Phenotypic comparison of Coronado(T) and other members of the same clade within the
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