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Thalassobaculum salexigens sp. nov., a new member of the family Rhodospirillaceae from the NW Mediterranean Sea, and emended description of the genus *Thalassobaculum*

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Abstract:

A novel Gram-negative bacteria, named CZ41_10a^T, was isolated from coastal surface waters of the north-western Mediterranean Sea. Cells were motile, pleomorphic rods, 1.6 µm long and 0.7 µm wide and formed cream colonies on marine agar medium. The G+C content of the genomic DNA was 65 mol%. Phylogenetic analysis of 16S rRNA gene sequences placed the new isolate in the genus *Thalassobaculum*, a member of the family *Rhodospirillaceae*, class *Alphaproteobacteria*. Unlike *Thalassobaculum litoreum* CL-GR58^T, its closest relative, strain CZ41_10a^T was unable to grow anaerobically and did not exhibit nitrate reductase activity. On the basis of DNA–DNA hybridization, fatty acid content and physiological and biochemical characteristics, this isolate represents a novel species for which the name *Thalassobaculum salexigens* sp. nov. is proposed. The type strain is CZ41_10a^T (=DSM 19539^T=CIP 109064^T=MOLA 84^T). An emended description of the genus *Thalassobaculum* is also given.

Bacteria performing the many steps of the nitrogen cycle are of great interest for agricultural and environmental questions. Among these organisms, members of the genus *Azospirillum* are well studied because of their association with plants and their ability to fix nitrogen. Eleven species have already been described. Three strains, close to *Azospirillum* species, were isolated from surface waters in the Mediterranean Sea. As they were putative new taxa, an investigation into their taxonomy and characteristics were performed. Their taxonomic position and their environmental origin led to hypotheses of their possible involvement in the nitrogen cycle, as the marine nitrogen cycle and nitrogen flows between oceans and the atmosphere are key processes (Capone & Knapp, 2007). Of the three strains, two have been recently described as members of a novel genus, *Nisaea* (Urios *et al.*, 2008). Denitrification genes *nir*K and *nos*Z were found in *Nisaea denitrificans* whereas *Nisaea nitritireducens* possessed only *nir*K. The ability to denitrify was confirmed by culturing methods. The third strain, named CZ41_10a^T, was not able to denitrify and did not possessed *nir*K nor *nos*Z. CZ41_10a was phylogenetically placed between the genera *Nisaea* and *Azospirillum*. Recently, *Thalassobaculum litoreum*, a new species of a new genus belonging to the family *Rhodospirillaceae* was published (Zhang *et al.*, 2008). The type strain shared 99% 16S rDNA gene sequence similarity with strain CZ41_10a^T but DNA-DNA hybridization, G+C content, fatty acid composition, physiological and biochemical characteristics showed numerous and important differences between *Thalassobaculum litoreum* and strain CZ41_10a^T. In this paper these different properties are provided and demonstrate CZ41_10a^T represents a new species of the genus *Thalassobaculum*.

Samples were collected in February 2004 at the SOLA station located in the bay of Banyuls-sur-Mer (42°29’N 3°08’E) at 3 m depth. Subsamples were spread on Nutrient Agar plates (BIO-RAD, Marnes-la-Coquette, France) made with filtered sea water, and incubated at 25°C

for 2 weeks. Colonies were picked and purified by 3 subcultures. Among these colonies, an isolate forming cream coloured colonies was obtained and referenced as strain CZ41_10a^T.

Microscope observations (Olympus AX70) showed that cells from isolate CZ41_10a^T were motile rods, approximately 1.6 ± 0.3 µm long and 0.7 ± 0.2 µm wide. Cells were negatively stained for transmission electron microscopy (Raguénès *et al.*, 1997). A single polar flagellum was observed. The Ryu KOH reaction (Powers, 1995) led to an immediate cell lysis that was confirmed by microscopy (Olympus AX70). This positive reaction indicated that the cells were Gram-negative.

The isolate was grown in marine broth medium (MB 2216, Difco, Detroit, Mich, USA). For determining its salinity range, marine broth medium was prepared according to the composition provided by the manufacturer but with different NaCl concentrations. For the pH range, MES, PIPES, AMPSO or MOPS (Sigma, St. Louis, MO, USA) was added to marine broth medium to achieve different pH values. Cultures were incubated at 30°C under aerobic conditions. Methods for the determination of growth parameters were as reported by Wery *et al.* (2001b). Growth was observed at 15-37°C with an optimum temperature of 30°C (Supplementary Fig. S2). The strain grew at sea salts concentrations ranging from 10 to 80 g l^{-1} (7 to 54 g l^{-1} NaCl) and an optimum concentration could be defined between 50 and 60 g l^{-1} (34 to 40 g l^{-1} NaCl) (Supplementary Fig. S2). No significant growth was observed without sea salts. Growth occured over a range pH 5.0 - 10.0 with a clear optimum at pH 8.0. Growth decreased by 50% at pH 9.0, compared to the value obtained at pH 8.0 whereas a relative decrease of only 22% was observed at pH 6.0 (Supplementary Fig. S2).

The strain's ability to use different substrates was investigated using Biolog GN2 MicroPlates (Tang *et al.*, 1998) according to the manufacturer's instructions except for the incubation

time: measurements were made hourly during 24h. Positive reactions were observed for arabitol, erythritol, fructose, fucose, glucose, lactose, lactulose, mannitol, γ-hydroxybutyrate and propionate (Table 1). Weak positive reactions were noted for adonitol, mannose, sorbitol and β-hydroxybutyrate (Table 1).

Enzymatic activities of the strain were investigated using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Leucine arylamidase and valine arylamidase exhibited positive reactions and a weak signal was observed for acid phosphatase (Table 1).

Fatty acid methyl esters composition was carried out by the Identification Service of the DSMZ (DSMZ, Braunschweig, Germany). The fatty acid compositions for strain CZ41_10a^T and related bacteria are shown in Table 2. The large amounts of 18:1 ω7c and, to some extent, 16:1ω7c and 16:0, were comparable to those known in *Nisaea denitrificans* DR41_21^{T}and *Thalassobaculum litoreum* CL-GR58^T. In contrast, 17:1 ω6c, 17:1 ω8c, 11 methyl 18:1 ω7c and 17:0-3OH were not found in *Nisaea denitrificans* DR41_21^T and 17:0 was 16-fold more represented than in *Nisaea denitrificans* DR41_21^T and *Nisaea nitritireducens* DR41_18^T. 20:1ω9c and the unknown fatty acid ECL 18.814 found in *Thalassobaculum litoreum* CL-GR58^T were not detected in strain CZ41_10a^T. The level of 19:0 cyclo ω8c in CZ41_10a^T is comparable to that of *Nisaea nitritireducens* DR41_18^T but 6-fold less than found in *Thalassobaculum litoreum* CL-GR58^T. 18:1ω7c and 16:1ω7c / i-15-2OH contents found in strain CZ41_10a^T are higher than in *Nisaea* strains but lower than in *Thalassobaculum litoreum* CL-GR58^T. Among the 4 major fatty acids found in strain CZ41_10a^T, 3 have significantly higher contents than in *Thalassobaculum litoreum* (18:1 ω7c, 17:0 and 16:1ω7c / i-15-2OH) and 16:0 is clearly less represented than in *Thalassobaculum litoreum* CL-GR58^T but has the same value than in *Nisaea* strains.

Analysis of respiratory quinones was carried out by the Identification Service of the DSMZ Identification Service (DSMZ, Braunschweig, Germany). Strain CZ41_10a^T has mainly ubiquinone-10 (Q 10).

Genomic DNA was extracted as described by Wery *et al.* (2001a). The G+C content was determined by thermal denaturation using the method of Marmur & Doty (1962) and conditions reported by Raguénès *et al.* (1997). The G+C content of the genomic DNA of strain CZ41_10a^T was 65 mol%. The 16S rRNA gene was amplified and sequenced as described by Agogué *et al.* (2005). The sequence was analysed as described by Urios *et al.* (2006). Strain CZ41_10a^T was phylogenetically affiliated to the family *Rhodospirillaceae* in the phylum *Proteobacteria* (Fig. 1). The nearest relatives were *Thalassobaculum litoreum* DSM 18839^T (similarity value of 99%), *Nisaea nitritireducens* (= DSM 19540^T) (similarity value of 94%) and *Nisaea denitrificans* (= DSM 18348^T) (similarity value of 93%). Consequently, DNA-DNA hybridization was performed by the DSMZ Identification Service (DSMZ, Braunschweig, Germany). The results indicated 66% ± 1 % of DNA-DNA similarity between *Thalassobaculum litoreum* DSM 18839^T and strain CZ41_10a^T.

Because of the metabolic capabilities of the closest relatives of strain CZ41_10a^T, participation to the nitrogen cycle was investigated. To amplify *narG, nirS, nirK, nosZ, nifH* and *AmoA* genes, published primers were used: na3F-narG5'R for *narG* (Goregues *et al.*, 2005), cd3F-cd4R for *nirS* (Michotey *et al.*, 2000), nirKCF-nirKCR for *nirK* (Goregues *et al.*, 2005), nosZ1211F-nosZ1897R for *nosZ* (Rosch *et al.*, 2002), nifHF-nifHR for *nifH* (Zehr & McReynolds, 1989), AmoA-1F-AmoA-2R for *AmoA* (Rotthauwe *et al.*, 1997). PCR amplification was carried out as previously described (Urios *et al.*, 2008). Among the tested genes, none were successfully amplified by PCR. Anaerobic growth experiments were

performed as described by Urios *et al.* (2008). Unlike *Nisaea denitrificans* and *Thalassobaculum litoreum* CL-GR58^T (Zhang *et al.*, 2008), no growth was observed.

Some characteristics of strain CZ41_10a^T are quite similar to those of *Thalassobaculum litoreum* CL-GR58^T, its nearest relative such as motile cells with a single polar flagellum, optimal growth temperature and pH, ubiquinone Q-10 and some of the major fatty acids. Nevertheless, strain CZ41_10a^T has a 12-fold higher salinity optimum and an inability to grow without sea salts, a wider pH growth range, a 3% lower G+C content, 6 out of 8 different API ZYM positive reactions and a fully different substrates profile. Unlike *Thalassobaculum litoreum* CL-GR58^T, strain CZ41_10a^T was not able to grow anaerobically, nor did it exhibit nitrate reductase activity. Finally, DNA-DNA hybridization results indicated 66% of DNA-DNA similarity.

Based on phenotypic and genotypic differences between strain CZ41_10a^T and its nearest described relatives, we propose that strain CZ41_10a^T should be assigned to a novel species belonging to the *genus Thalassobaculum*. Due to the sea salts requirement for the growth of strain CZ41_10a^T, the name *Thalassobaculum salexigens* sp. nov. is proposed.

Description of *Thalassobaculum salexigens* sp. nov.

Thalassobaculum salexigens (sa.lex'i.gens. L. n. sal salis salt, sea water; L. v. exigo to demand; N.L. part. adj. salexigens sea water-demanding). The strain forms cream colonies on Marine Agar medium. The G+C content of strain CZ41_10a^T is 65%. Ubiquinone is ubiquinone Q-10. Major fatty acids are: 18:1 ω7c, 17:0, 16:0 and 16:1ω7c. Growth occurs at 15 - 37°C (optimum 30°C), at pH 5.0 - 10.0 (optimum 8.0) and salinity range of 7 - 54 g l^{-1} (optimum 34 - 40 g l^{-1}). Catalase and oxidase tests were positive. Positive reactions with

Biolog GN2 plates were obtained for arabitol, erythritol, fructose, fucose, glucose, lactose, lactulose, mannitol, γ-hydroxybutyrate and propionate. API ZYM positive reactions for enzymatic activities were obtained for leucine arylamidase and valine arylamidase. The type strain CZ41_10a^T (= DSMZ 19539^T = CIP 109064^T = MOLA 84^T) was isolated from the water column in the bay of Banyuls-sur-Mer (42°29'N 3°08'E).

Emended description of the genus *Thalassobaculum* Zhang *et al.* 2008

The description of the genus *Thalassobaculum* is as given by Zhang *et al.* (2008) with the following amendments. Some of the strains are facultative anaerobes. The major fatty acids are 18:1ω7c, 16:0, 17:0 and summed feature 3 (16:1ω7c and/or iso-15:0 2-OH). DNA G+C content is 65-68 mol%.

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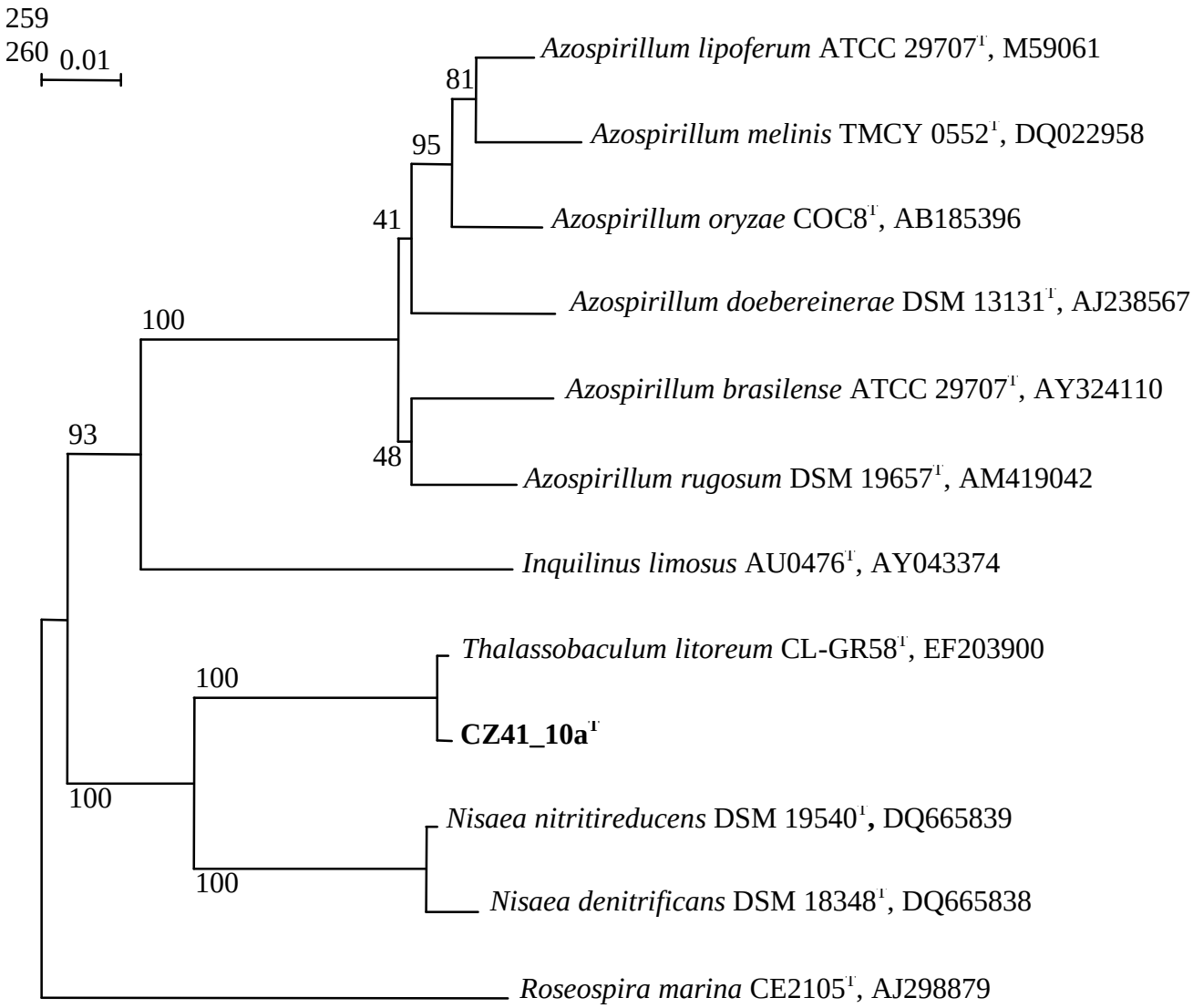
TABLE 1. Characteristics distinguishing strain CZ41_10a^T from related species.
Strains: 1, *Nisaea denitrificans* DR41_21^T; 2, *Nisaea nitritireducens* DR41_18^T; 3, strain CZ41_10a^T; 4, *Thalassobaculum litoreum* CL-GR58^T. +, positive; -, negative; w, weakly positive.

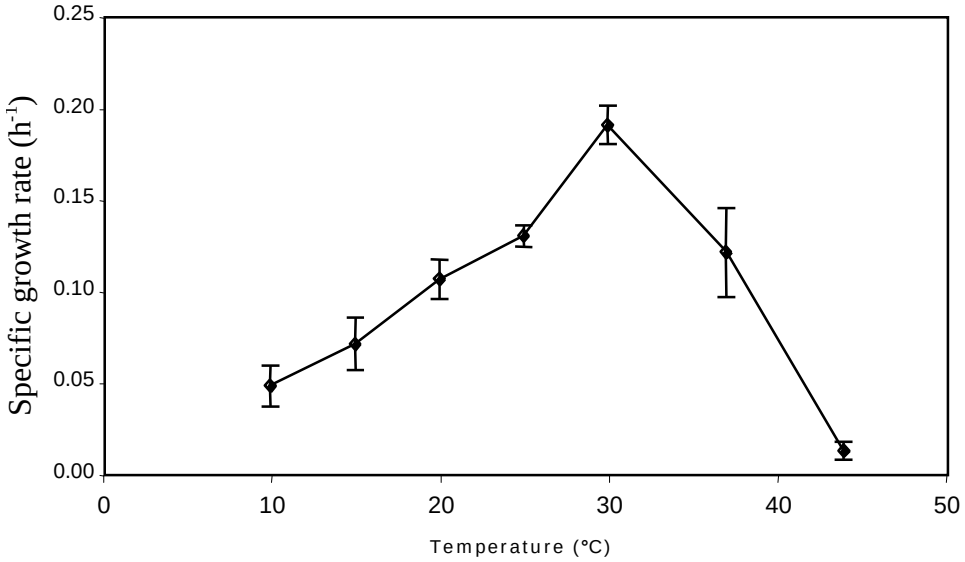
	1	**2**	**3**	**4**
G+C content (mol %)	60	60	65	68
Growth conditions				
température range (optimum) °C	15-(30)-44	15-(30)-44	15-(30)-37	10-(30-35)-35
salinity range (optimum) (g.L^{-1})	0-(20)-60	0-(20)-60	7-(34-40)-54	1-(2-4)-10
pH range (optimum)	5-(6)-9	5-(6)-9	5-(8)-10	7-(8)-9
Substrates:				
ribose	-	-	-	+
arabinose	-	-	-	+
fructose	+	+	+	-
glucose	+	+	+	-
trehalose	w	w	-	-
lactose	-	-	+	-
maltose	-	-	-	ND
mannitol	w	w	+	-
glycerol	w	w	-	ND
xylitol	w	w	-	ND
raffinose	+	+	-	-
sucrose	-	-	-	+
acetate	+	+	-	-
γ-hydroxybutyrate	+	+	+	ND
propionate	+	+	+	ND
API ZYM reactions:				
alkaline phosphatase	+	+	-	+
leucine arylamidase	+	+	+	+
valine arylamidase	-	-	+	+
acid phosphatase	+	+	w	+
esterase (C4)	-	-	-	+
naphtol-AS-BI-phosphohydrolase	-	-	-	+
α- & β-glucosidases	-	-	-	+
N-acetyl-β-glucosaminidase	-	-	-	+

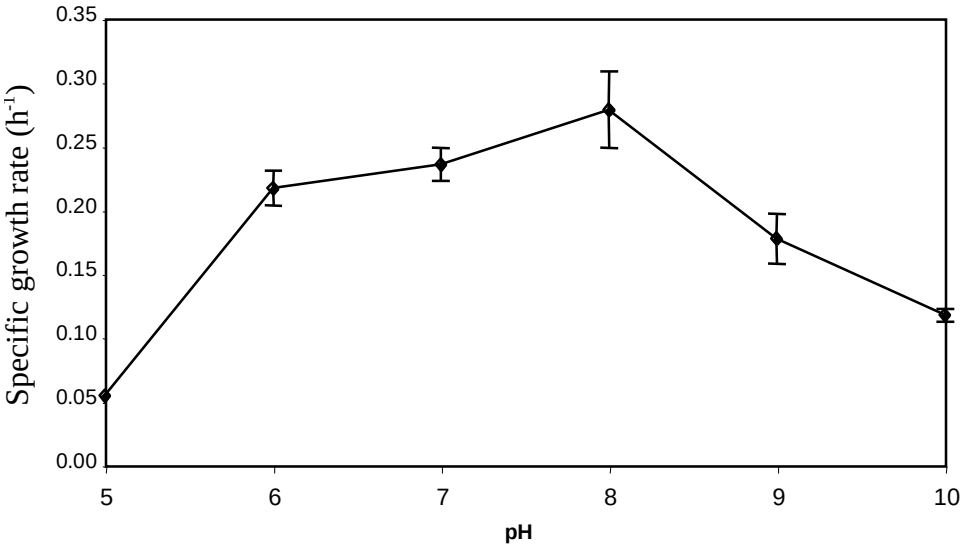
TABLE 2. Comparison of lipids compositions of strain CZ41_10a^T and related species. Strains: 1, *Nisaea denitrificans* DR41_21^T; 2, *Nisaea nitritireducens* DR41_18^T; 3, strain CZ41_10a^T; 4, *Thalassobaculum litoreum* CL-GR58^T. tr, traces. All strains were grown on MA2216.

	1	**2**	**3**	**4**
16:0 3OH	1.1	1.0	-	-
17:0 3OH	-	-	1.1	tr
18:0-3OH	-	1.0	1.0	tr
15:0	1.0	-	1.7	1.5
16:0	11.3	10.6	10.7	14.8
17:0	1.0	1.0	16.0	12.2
18:0	-	1.0	1.1	2.0
17:1ω6c	-	-	1.3	tr
17:1ω8c	-	-	1.1	tr
18:1 ω7c	69.1	67.6	55.4	48.5
16:1ω7c / i-15-2OH	13.9	14.1	8.1	6.0
10-methyl 19:0	1.0	-	-	-
11 methyl 18:1 ω7c	-	-	2.1	3.0
19:0 cyclo ω8c	-	1.8	1.0	6.3
20:1ω9c	-	-	-	tr
ECL 18.814	-	-	-	1.4


259
260
0.01
81
95
41
100
93
48
100
100
100
100
Azospirillum lipoferum ATCC 29707^{T}, M59061
Azospirillum melinis TMCY 0552^{T}, DQ022958
Azospirillum oryzae COC8^{T}, AB185396
Azospirillum doebereinerae DSM 13131^{T}, AJ238567
Azospirillum brasilense ATCC 29707^{T}, AY324110
Azospirillum rugosum DSM 19657^{T}, AM419042
Inquilinus limosus AU0476^{T}, AY043374
Thalassobaculum litoreum CL-GR58^{T}, EF203900
CZ41_10a^{T}
Nisaea nitritireducens DSM 19540^{T}**,** DQ665839
Nisaea denitrificans DSM 18348^{T}, DQ665838
Roseospira marina CE2105^{T}, AJ298879

260
261
262


Specific growth rate (h-1)
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
Temperature (°C)


Specific growth rate (h-1)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5
6
7
8
9
10
pH

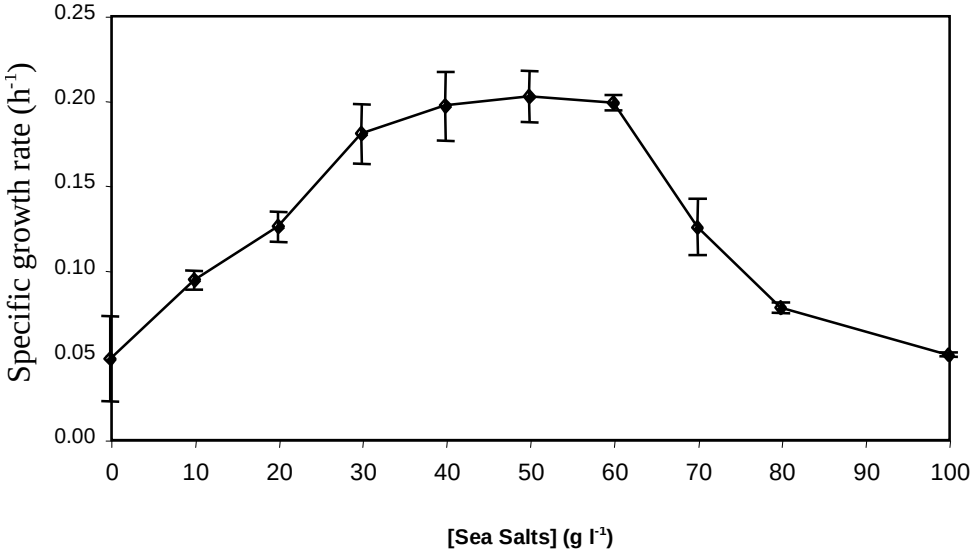

Specific growth rate (h-1)
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
80
90
100
[Sea Salts] (g l-1)

Fig. 1. Phylogenetic tree showing the position of strain CZ41_10a^{T}. *Roseospira marina* was used as an outgroup. Accession numbers and type strains are indicated. The tree corresponds to an unrooted tree obtained by the neighbor-joining algorithm (Kimura corrections). Bootstrap values are displayed on their relative branches.

Supplementary figure :

Fig. S2. Effects of temperature, pH and NaCl concentration on the growth of strain CZ41_10a^{T}.