Identification of marine bacteria from the Swedish nature reserve Kullaberg by the 16S rRNA gene: abundance in novel species and genera

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Summary

The bacterial communities in the surrounding waters of Kullaberg nature reserve in Southern Sweden were investigated by means of cultivation and 16S rRNA gene sequence analysis. Samples were collected from three localities and six different depths (in March 2008) to allow us to explore the possible differences in cultivable bacterial diversity and community structure within this marine environment. A total of 214 sequences were analyzed, with the number of sequences obtained ranging from 48 to 97 based on locality. Almost three times as many sequences were obtained from bottom samples compared to the average numbers for the other depths. The number of species that could be linked to known type strains ranged from 37 to 71% based on depth, while the differences between the different localities (52-

58%) were not as distinct. Most species found belonged to the order *Bacillales,* the α and γ subclass of *Proteobacteria* and the *Cytophaga–Flavobacterium–Bacteroides* group. Major genera found within these groups include *Bacillus*, *Colwellia*, *Psychrobacter*, *Algibacter* and *Flavobacterium.* Notably, nearly half of the identified sequences turned out to belong to either a novel species or genus.

Introduction

The rocky Kullaberg peninsula in Southern Sweden forms the northeastern boundary for the Øresund strait (The Sound); the second largest water passage connecting the Baltic Sea to the North Sea. The area gained the status of natural reserve in 1965 and 1971 (Eastern and Western part) and by 1986 the reserve was widened to include the surrounding waters out to a distance of 300 meters from the mainland. Kullaberg was the first piece of land that arose in Sweden after the last ice age and parts of it have been uncultivated since 7500 B.C when the first leafed trees immigrated from the south (Påhlson, 2003). The nature reserve inhabits 70% of all the plant species recorded in Sweden creating a unique environment, which was the reason for making a microbial inventory of Kullaberg and its surrounding waters.

The marine environment is characterized by constantly changing conditions caused by the large influx of heavy salt water from the north and the accompanying outflow of brackish water from the south. This creates a salinity and thus also density gradient marked by a more or less defined halocline throughout the year (Hinrichsen *et al.*, 2001). Another important factor that may influence the environment is the heavy shipping, serving the whole of the Baltic region, which occurs in close proximity throughout most of the strait (Strand *et al.*, 2003).

The bacterial community in similar environments has been shown to shift in composition in response to the transition from saltwater to brackish water (Henriques *et al.*, 2006), while seasonal changes in temperature affects both the community structure and growth rate (Autio, 1998; Pinhassi *et al.*, 1997; Wikner & Hagstrom, 1991). Together these abiotic factors create a dynamic environment that should influence the bacterial community structure in turn. In an effort to investigate the composition of the

cultivable bacterial community, samples were collected during the late winter months from different depths (from the bottom to the surface), at three different localities covering the outmost western tip and the accompanying north and south near shore waters of Kullaberg. Identification of the bacteria through isolation and cultivation enables us to describe them as novel species and genera and to use them in future applications.

Material and methods

Sampling and cultivation

From the three different localities in the surrounding waters of Kullaberg in southern Sweden (in March 2008) a total of 18 samples were collected. At each locality sampling were performed on shallow and deep bottom and open water at depths of between 5 - 7 meters and 16 - 24 meters and also on the halocline and surface water making a total of 6 samples. The samples were collected in separate 15 ml plastic tubes, either from boat (surface samples) or by the aid of scuba gear (all remaining samples). Free water samples were collected by simply opening the plastic tubes in the water column and resealing them after they had filled up completely. Bottom samples were collected with the aid of a sharp, circular metal tool with a diameter of 1.0 cm (Olofsson *et al.*, 2007), which were driven by hand into the sediment. Core samples were then ejected into the plastic tubes using a sharp pin to empty the tool.

The samples were immediately transported by car, in ambient temperature, to the laboratory in Helsingborg, 40 km from Kullaberg. Dilution was made from 0.1 ml of each sample in a ten-fold dilution series, containing physiological saline (0.9% w/v NaCl, 0.1% w/v Tween 80 and 0.1% w/v peptone), for viable count. From each dilution 0.1 ml was spread on Marine agar 2216 (Difco) surface plates and incubated aerobically at 22° C for 72 h.

The number of colony forming units (cfu) for each plate where counted under magnification and colonies where selected for pure culture identification based on visual characterization so that at least one distinct colony morphology was obtained.

PCR of isolates

One of the colonies from the purified isolates was placed in a 0.2 Thermo-Strips (Abgene, Surrey, UK) together with glass beads (0.106 mm, Sigma-Aldrich, St Louis, MO, USA) in 0.1 ml sterile water. The cells were disintegrated by being shaken for 45 min in an MS1 Minishaker (IKA Works, Wilmington, DE, USA). After centrifugation, 20 200 g for 5 min in a Galaxy mini-centrifuge (VWR, West Chester, Pennsylvania, USA), 1 µl of the supernatant was used in the PCR reaction that followed**.**

Amplification was conducted using primers designed to anneal to conserved regions of bacterial 16S rRNA genes. The forward primer ENV1 (5′-AGA GTT TGA TII TGG CTC AG- 3′) corresponded to positions 8-27 in *Escherichia coli* 16S rRNA, the reverse primer ENV2 (5′-CGG ITA CCT TGT TAC GAC TT- 3′) corresponding to positions 1511-1492 (Brosius *et al.*, 1978). The PCR reaction contained 5 µl 10 x PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 200 µmol l^{-1} of each deoxyribonucleotide triphosphate, 2.5 U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 10 pmol of each primer and 1-10 µl template in a total volume of 50 µl. Amplification was performed using a Mastercycler (Eppendorf, Hamburg, Germany) as follows: 30 cycles at 95°C for 15 s, 48°C for 30 s and 72°C for 90 s followed by an elongation step at 72°C for 10 min. The PCR product was stored at –20°C for sequencing.

Sequencing and identification of isolates

PCR products originating from the bacterial samples were sequenced by a sequencing company (MWG Biotech, Ebersberg, Germany) using the universal primers ENV1 and ENV2. These 16S rDNA sequences were searched against GenBank (National Centre for Biotechnology Information, Rockville Pike, Bethesda, MD) using the Advanced BLAST similarity search option (Altschul *et al.*, 1997), accessible from the homepage of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The 16S rRNA gene sequences were also checked using the software RDP (Ribosomal Database Project II) (Cole *et al.*, 2005), accessible from the homepage (http://rdp.cme.msu.edu/). The closest related type strains found were gathered for comparison. The compared sequences were between 141 and 1055 bp in length.

Phylogeny

The phylogenetic tree shown in fig. 2-4 was obtained using computer software programs Clustal X (version 1.81) (Thompson *et al.*, 1997) for alignment, BioEdit (version 6.0.7) (Hall, 1999) for editing, and PAUP (version 4.0 beta) (Swofford, 2003) for computing the phylogenetic tree. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) in PAUP by Distance Matrix. Evolutionary distances were estimated using the LogDet/Paralinear method. Bootstrap values were computed using 100 to 1,000 re-samplings, evolutionary distances being estimated using the LogDet/Paralinear method. The 16S rRNA gene sequences generated in this study are available from GenBank under the accession numbers: KC462889 - KC463011 which are displayed in the phylogentic trees in fig. 2-4.

Results

A total of 260 pure cultivated isolates were sequenced, with 66 (25.4%) originating from the South, 111 (42.7%) from the tip and 83 (31.9%) from the North sampling area. 224 sequences were of sufficient length for further evaluation. After aligning and grouping identical sequences, 137 unique sequences were retrieved with 87 matching copies (10 sequences were excluded from the results either because of insufficient length or inconsistent results during the aligning process). All except two showed a similarity of >90% to sequences in RDP and GenBank.

Distribution of genera

Sequences from the three different localities were compared based on similar sample origin (depth) for comparison of the general distribution of genera (Fig. 1). Shallow water samples (Fig. 1c) comprising 16 genera and shallow bottom samples (Fig. 1d) comprising 21 genera showed the highest number of diversity at the genus level. *Colwellia* which was found as 10 isolates in shallow water samples and 19 isolates in deep water samples, were the dominant genus in both water and bottom samples. *Phaeobacter* and *Psychrobacter* were both found in shallow water samples at elevated numbers (7 and 4 respectively) but not found in shallow bottom samples. *Flavobacterium* found as 8 isolates in shallow bottom samples

were only retrieved as a single isolate in shallow water. *Algibacter* was also found at both depths with the highest number in the bottom samples.

Samples from deep water (Fig. 1e) containing 11 genera and deep bottom (Fig. 1f) containing 14 genera were dominated by *Psychrobacter* and *Bacillus* (7 and 39 isolates respectively); neither genus was found at the opposite depth. *Colwellia* were the second most common genus in both locations with 3 and 5 isolates found in deep water and deep bottom isolates respectively.

Identification of sequences

The distribution of sequence similarity to previously described type strains were found to vary widely between different depths; the results are depicted in table 1. Shallow water and deep bottom samples amounted to 59% of the total number of isolates found. While samples collected at the surface and in the vicinity of the halocline contained the least number of cultivable isolates (17%) as can be seen in table 1.

Phylogeny

The phylogenetic trees in fig. 4 to 6 represent the bacterial composition found within each locality based on sequence similarity to type strains.

Discussion

The bacterial 16S rRNA gene sequences, obtained from marine bacterial isolates in this survey, were distributed over 43 different genera; almost half of these genera came from single isolates. Only five genera (*Bacillus* [42], *Colwellia* [37], *Psychrobacter* [15], *Algibacter* [14] and *Flavobacterium* [11]) (number of isolates found within brackets) contained more than ten isolates each; together they made up 56% of the bacteria found.

Bacteria that belonged to previously described type strains, based on a similarity greater than 97% (Stackebrandt & Goebel, 1994) consisted of totally 55% and varied from as low as 37 and 38% for

shallow bottom and shallow water samples, up to 71% for surface and deep bottom samples (Table 1) The difference between the three localities was not as distinct as previously described with identifiable species ranging from 52 to 58% similarity (Table 1). Sequences with closest similarity to a previously described type strain between 97 to 95% (29%) and 95 to 91% (15%) most probably belong to novel species or genera respectively (Wolfgang *et al.*, 1998). Furthermore, it is possible that sequences with a similarity between 98.5 and 97 (19%) may as well belong to novel species (Table 1).

The actual number of identified isolates obtained varied markedly from 97 sequences at the tip to 48 sequences at the south sampling site. Most sequences (63 and 64) were obtained in shallow and deep bottom samples, while the smallest amount of sequences (14 and 22) was obtained at the surface and within the halocline (Table 1).

Bacterial community composition

The sequenced bacteria were distributed among three major and one minor phylogenetic groups after the alignment. These include the order *Bacillales* (Cluster I in Fig. 2-4), classes Gamma *Proteobacteria* (Cluster II in Fig. 2-4) and Alpha *Proteobacteria* (Cluster III in Fig. 5-6) and also the *Cytophaga–Flavobacterium–Bacteroides* group (Cluster IV in Fig. 2-4).

Bacteria belonging to the spore forming genus *Bacillus* were exclusively found in shallow bottom (Fig. 1d) and deep bottom samples (Fig. 1f) indicating that they were associated with the sediment. The four most common species found were *Bacillus hwajinpoensis, Bacillus muralis, Bacillus weihenstephanensis and Bacillus algicola*, that constituted nearly three quarters of the 42 isolates linked to *Bacillus*. Moreover, these isolates showed a high level of sequence similarity (>99.1%) to known type strains (Cluster I in Fig. 2-4). Notably, *B. muralis* was first isolated from mural paintings in Germany and described to have the ability to reduce nitrate to nitrite and hydrolyze starch (Heyrman *et al.*, 2005). *B.weihenstephanensis* is a psychrotolerant toxin producer which belongs to the Bacillus cereus group (Baron *et al.*, 2007) known to cause food poisoning in humans (Mahler *et al.*, 1997). *B. algicola* was first isolated from the Brown Algae *Fucus evanescens* which is an invading species of arctic origin common in the area, and found to be alginolytic (Ivanova *et al.*, 2003; Wikstrom *et al.*, 2002).

178 Isolates within *Colwellia* were found at all sampling depths except at the surface (Fig. 1b-f). The highest
179 density was found in shallow water exhibiting 10 isolates and in bottom samples containing 19 isolates.
180 In contrast to the *Bacillus* sequences, all *Colwellia* sequences had a much lower sequence similarity
181 compared to known type strains, with a range from 97.8 to 93 percent. Three major groupings with the
182 closest similarity to species *Colwellia aestuarii, Colwellia piezophila* and *Colwellia psychrerythraea*
183 were clearly visible during the alignment (Cluster II in Fig. 2-4). *C. psychrerythraea* is considered a
184 model species for the study of life in cold environments, with optimal growth yield below the freezing
185 point (Methe *et al.*, 2005). It is also important from an ecological viewpoint since it has been shown to
186 produce a large number of cold stabilized extracellular enzymes, which are capable of degrading
187 proteins and peptides (Huston *et al.*, 2004). This could point to an important role for carbon flux not
188 only in a strictly arctic climate but also in temperate environments such as the one studied here.

189 Bacteria within the genus *Psychrobacter* (Fig. 1a-c, e) were found to be a major constituent of all pooled
190 free water samples. As evident from the name, bacteria within *Psychrobacter* are psychrophilic (or at
191 least psychrotolerant) , with many species found in cold environments (Bozal *et al.*, 2003; Heuchert *et*
192 *al.*, 2004; Jung *et al.*, 2005). In this study, the fifteen isolates found to belong to *Psychrobacter,* ranged
193 from 98 to 100 % similarity to known type strains distributed over six different species (Cluster II in
194 Fig. 2-4). One of the dominating *Psychrobacter*, *P. nivimaris,* was first isolated from the surface of
195 organic particles (i.e. marine snow) in the South Sea (Heuchert *et al.*, 2004). Another group of isolates
196 were closely related to the species *P. cibarius* which was first isolated from fermented Korean seafood
197 (Jung *et al.*, 2005).

198 The genus *Pseudoalteromonas* was another major component of the genera found in this study with 9
199 isolates (Cluster II in Fig 2-4). *Pseudoalteromonas* is a well known group that is usually isolated from
200 marine environments and of interest for, among others, their antifouling properties (Ivanova *et al.*,
201 2002).

202 One major but diffuse grouping encountered at all three localities (part of Cluster IV in Fig. 2-4) was
203 found to resemble the genus *Algibacter* with a similarity of almost 98 % (although 2 isolates fell below

93% resemblance). This genus was first described in 2004 and contains two species at the moment, *Algibacter lectus* which is a gliding, facultative anaerobic, heterotroph isolated from green algae found in the Sea of Japan and the recently described *Algibacter mikhailovii* which has been shown to decompose algal compounds such as agar and alginate (Nedashkovskaya *et al.*, 2004; Nedashkovskaya *et al.*, 2007).

Isolates resembling *Flavobacterium* were found mainly in pooled shallow bottom samples (Fig. 1d). Ten out of a total of eleven isolates had a similarity percentage in the range 92-95 % (Cluster IV in Fig 2-4), which may indicate a novel genus (Wolfgang *et al.*, 1998).

Two sequences within the *Alpha Proteobacteria* group from the western tip (Cluster III in Fig. 5) showed a similarity of 99 and 97.7% to *Staleya guttiformis* (Spesp4) and *Sulfitobacter brevis* (Spedv7). Notably, both *S. guttiformis and S. brevis* were originally isolated from the hypersaline Ekho lake in Western Antarctica (Labrenz *et al.*, 2000). One common denominator for the marine environment of Kullaberg and these two Antarctic lakes is that they are meromictic (containing non intermixing layers of water) over most parts of the year. Furthermore, the sequence Spesp4 was also found within the vicinity of the halocline.

Conclusions

As previously discussed, most sequences (97) were isolated from the western tip, compared to 69 and 48 for the north and south sampling locations. This in turn amounts to the fact that almost three times as many possibly novel species where found at the tip compared to the south (Table 1). This could be explained by environmental differences as the tip is the most dynamic and fluctuating one (on a yearly basis) out of three localities studied. The apparent richness in diversity could indicate that this locality represent bacteria from both the Baltic brackish water as well as the Northern oceanic communities to a higher extent compared to the other two localities. Notably, more than 60% of the sequences derived from shallow bottom and shallow water isolates had a sequence similarity below 97% and thus either belongs to a novel species or genus (Wolfgang *et al.*, 1998).

None of the major groups described so far are unexpected based on the properties of the type strains that they resemble, i.e. psychrophilic species or degraders of algal and organic matter. What is really of interest is the high diversity found within the isolates (Fig 1a-f) as well as the number of possibly novel species found (Table 1). Totally, 96 sequences, of the 214 sequences analyzed, turned out to belong to unknown species or genera, highlighting the necessity of further work within this area.

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Table 1. Total number and percentage of identified isolates from each depth (surface, halocline, shallow water, deep water, shallow bottom and deep bottom) and locality (south, tip, north) classified according to percentage of similarity to known type strains.

Sequence similarity	Number of isolates from each depth						Number of isolates from each locality			Total
	Surface	Halocline	Shallow water	Deep water	Shallow bottom	Deep bottom	North	Tip	South	
>98.5	9 (53%)	8 (57%)	10 (27%)	11 (50%	5 (8%)	35 (55%)	28 (41%)	35 (36%)	15 (31%)	78 (36%)
<98.5 and >97	3 (18%)	1 (7%)	4 (11%)	4 (18%)	18 (29%)	10 (16%)	11 (16%)	16 (16%)	13 (27%)	40 (19%)
<97 and >95	3 (18%)	5 (36%)	17 (46%)	4 (18%)	21 (33%)	11 (17%)	22 (32%)	29 (30%)	10 (21%)	61 (29%)
<95 and >91	2 (12%)		6 (16%)	3 (14%)	17 (27%)	6 (9%)	7 (10%)	16 (16%)	9 (19%)	32 (15%)
<91					2 (3%)	2 (3%)	1 (1%)	1 (1%)	1 (2%)	3 (1%)
Total	17	14	37	22	63	64	69	97	48	214

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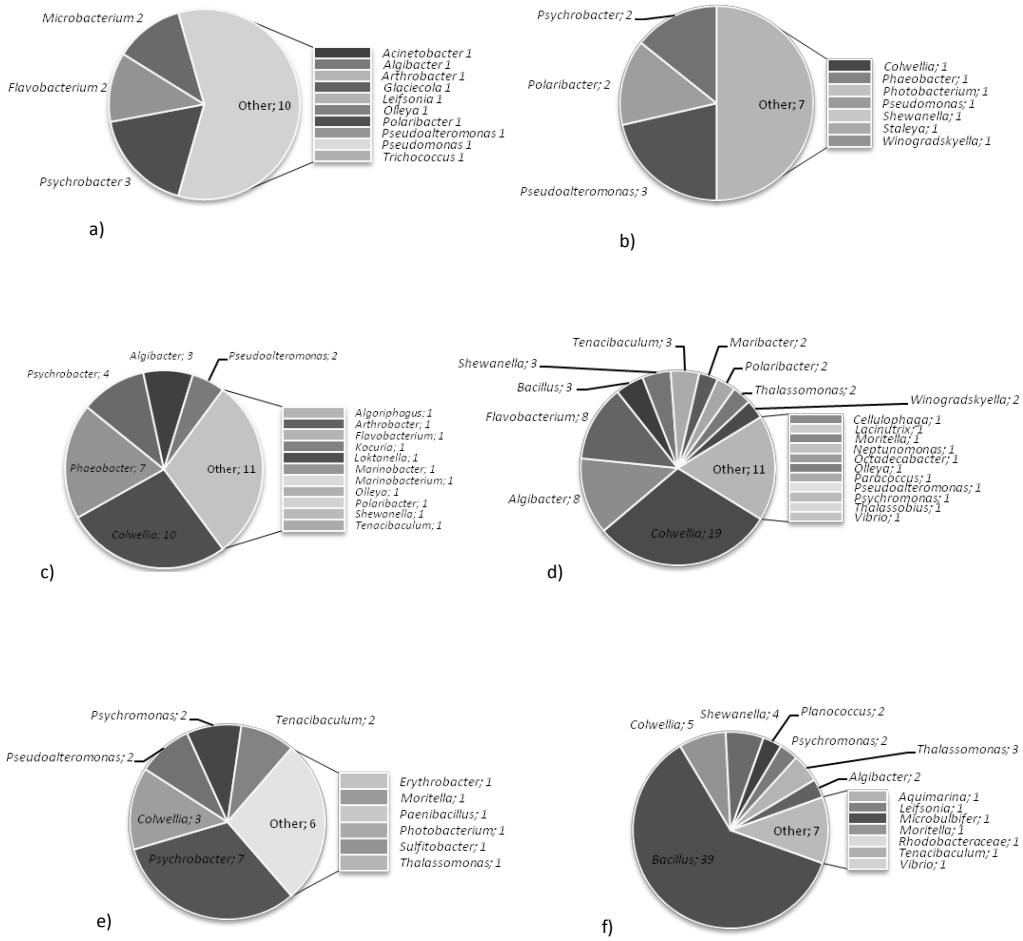

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363 **Fig. 1.** Distribution of bacterial genera for pooled samples based on a total of 17 surface isolates (a), 14 halocline
364 isolates (b), 37 shallow water isolates (c), 63 shallow bottom isolates (d), 22 deep water isolates (e) and 64 deep
365 bottom isolates (f).

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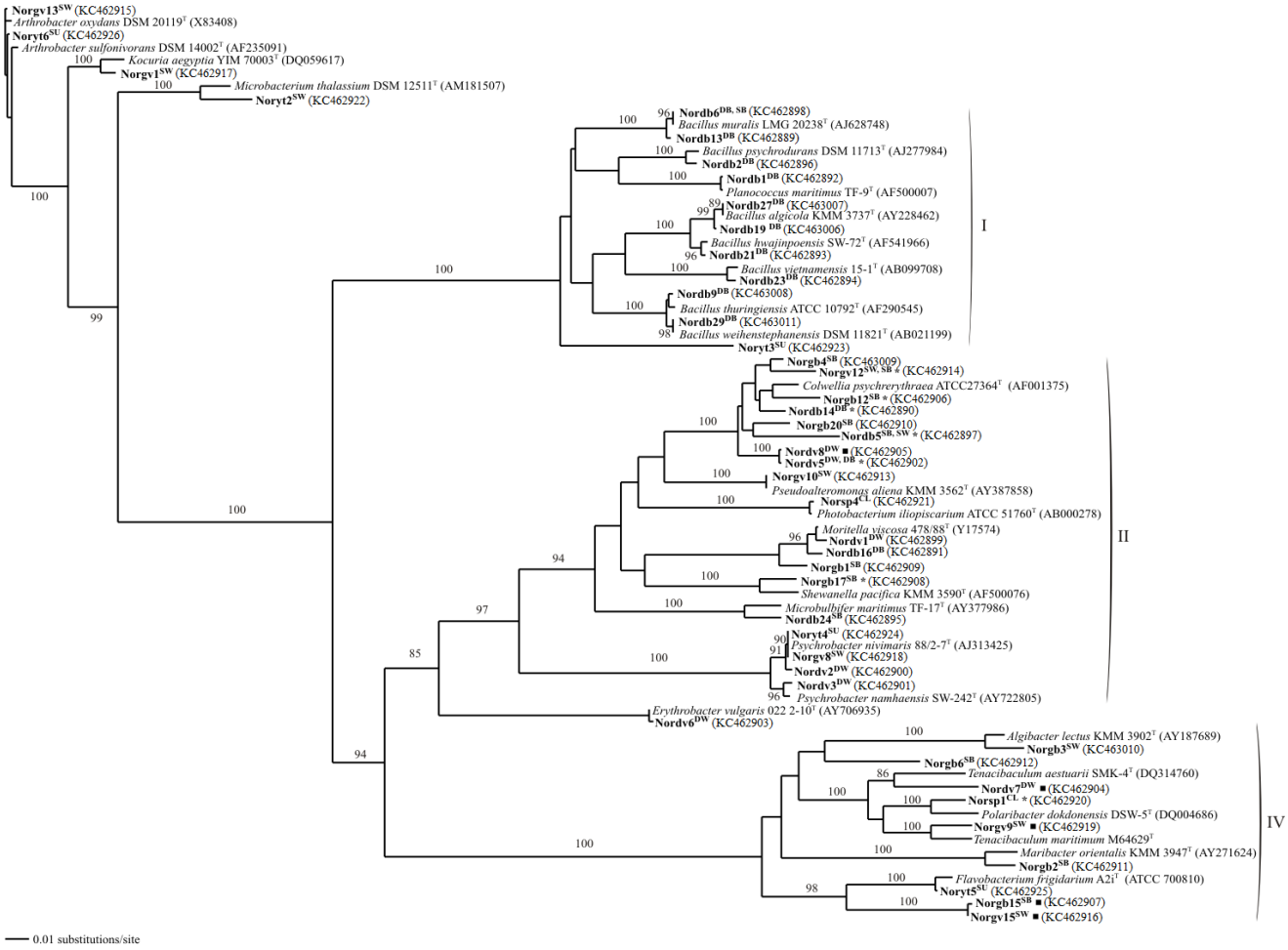

Fig. 2. Phylogenetic tree based on distance matrix analysis of 642 positions in the 16S rRNA gene of the unique sequences found at the North sampling location. Closely related type strains from RDP are included and indicated in parenthesis, together with accession numbers from GeneBank. Cluster I displays the *Bacillales* group, cluster II the Gamma Proteobacteria group and cluster IV the *Cytophaga–Flavobacterium–Bacteroides* group. Sequences with a similarity on genus level (<95 to >91%) or species level (<97 to >95%) are shown with a square or star (■,*). Bootstrap values of 85 percent and more from 1000 resamplings of the data set are given at the nodes. Bar: 0. 01 substitutions per nucleotide position.

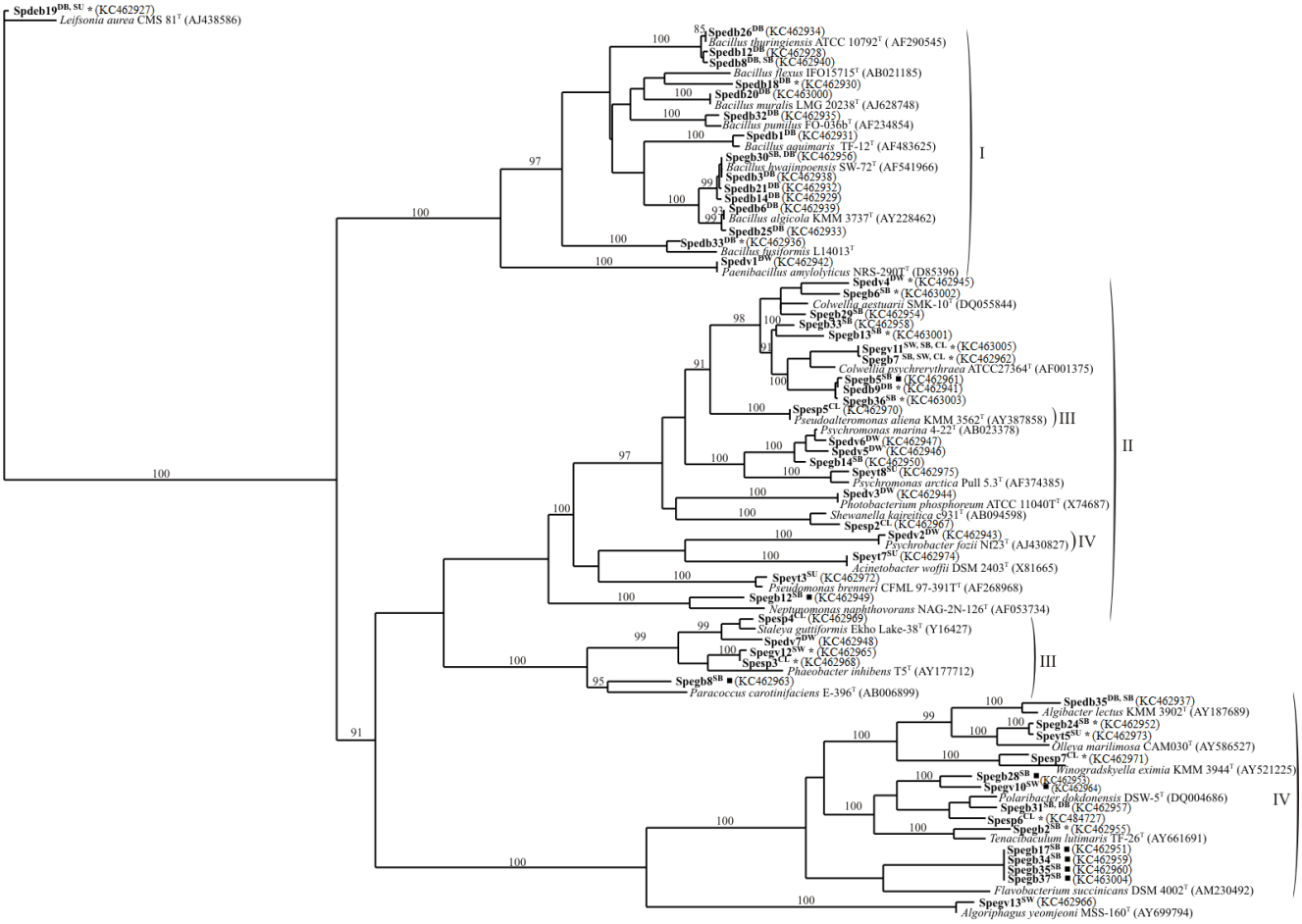

Fig. 3. Phylogenetic tree based on distance matrix analysis of 638 positions in the 16S rRNA gene of the unique sequences found at the Western (tip) sampling location. Closely related type strains from RDP are included and indicated in parenthesis, together with accession numbers from GeneBank. Cluster I displays the *Bacillales* group, cluster II the Gamma Proteobacteria group, cluster III the Alpha Proteobacteria group, and cluster IV the *Cytophaga–Flavobacterium–Bacteroides* group. Sequences with a similarity on genus level (<95 to >91%) or species level (<97 to >95%) are shown with a square or star (■,*). Bootstrap values of 85 percent and more from 100 resamplings of the data set are given at the nodes. Bar: 0. 01 substitutions per nucleotide position.

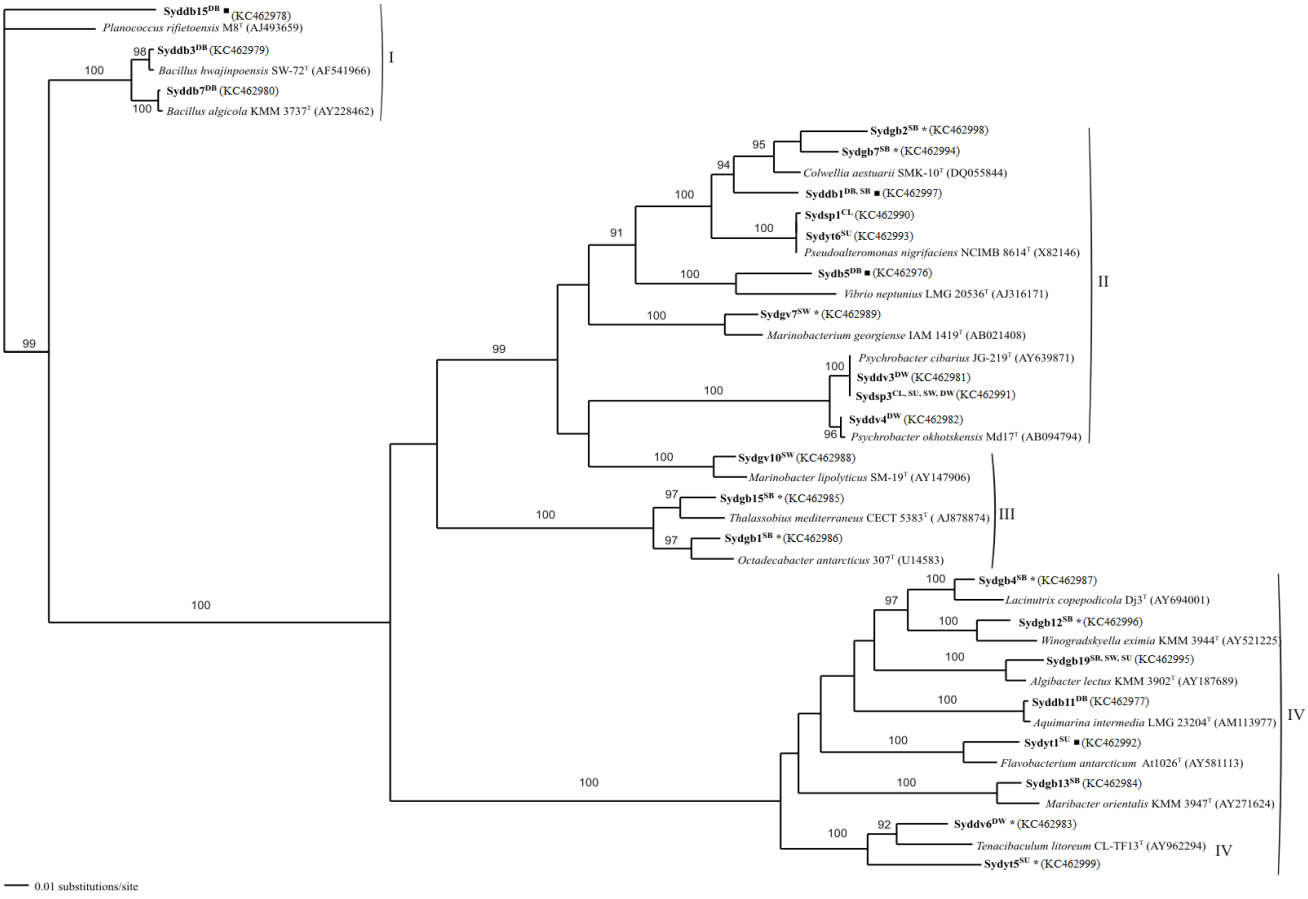

Fig. 4. Phylogenetic tree based on distance matrix analysis of 602 positions in the 16S rRNA gene of the unique sequences found at the South sampling location. Closely related type strains from RDP are included and indicated in parenthesis, together with accession numbers from GeneBank. Cluster I displays the *Bacillales* group, cluster II the Gamma Proteobacteria group, cluster III the Alpha Proteobacteria group, and cluster IV the *Cytophaga–Flavobacterium–Bacteroides* group. Sequences with a similarity on genus level (<95 to >91%) or species level (<97 to >95%) are shown with a square or star (■,*). Bootstrap values of 85 percent and more from 1000 resamplings of the data set are given at the nodes. Bar: 0.01 substitutions per nucleotide position.