Heterotrophic bacteria from brackish water of the southern Baltic Sea: biochemical and molecular identification and characterisation*

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Abstract

Six bacterial strains isolated from the surface water of the southern Baltic Sea were described on the basis of their morphological, physiological and biochemical features, and were classified on the basis of 16S rDNA sequence analysis. Comparative analyses of the 16S rDNA sequences of five of the six bacterial strains

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examined displayed a $\geq 98\%$ similarity to the sequences available in the NCBI GenBank. The 16S rDNA sequence of strain 2 shared only a 96% similarity with other published sequences, which suggests that this is a new, hitherto unknown species. The isolated heterotrophic bacteria belong to the families *Bacillaceae* (strain 1), *Flexibacteriaceae* (strain 2), *Sphingomonadaceae* (strains 3, 5), *Micrococcaceae* (strain 4) and *Aurantimonadaceae* (strain 6).

This is the first study in which the polyphasic approach has been applied to the identification of heterotrophic bacteria from the brackish waters of the Gulf of Gdańsk and Gdańsk Deep.

1. Introduction

The Baltic Sea is the largest brackish water system in the world. It is connected to the North Sea only through narrow and shallow straits between Denmark and Sweden. This outlet consists of a series of basins separated by shallow sills that obstruct efficient water exchange. Consequently, it takes 25–35 years for all the water from the Baltic Sea to be replenished by water from the North Sea and beyond.

The environmental conditions of the Baltic Sea are defined by the fresh water input from rivers and precipitation, and by the limited inflow of saline water from the North Sea. Without the constant, albeit small, influx of this saline water through the Danish Straits, the Baltic Sea would have been transformed into a gigantic fresh water lake long ago. There is a distinct salinity gradient from the almost oceanic conditions in the northern Kattegat to the almost fresh water conditions in the northern Bothnian Bay. A salinity barrier also exists between the surface and the bottom of the Baltic. Saline water, naturally heavier than fresh water, flows along the seabed. The fresh water on the surface of the sea does not mix appreciably with the saline water underneath.

The semi-enclosed and sensitive Baltic Sea is affected by a combination of unfavourable natural conditions – the shape of the seabed, the limited water exchange with adjacent seas, the formation of salinity and temperature barriers between surface water and bottom water – and constant pressure from pollution and human overexploitation. The Baltic Sea is unique in that there are areas where freshwater, brackish and marine species are all present together. This regularity also holds true for Baltic heterotrophic bacterioplankton. There are approximately $2–4 \times 10^6$ bacterial cells per millilitre throughout the water column in the Baltic Sea (Heinänen & Kuparinen 1991). Our investigations indicated $0.3–2.1 \times 10^6$ bacterial cells per millilitre. Despite the fact that this number of marine bacteria has been known for a dozen or so years at least, it is still not known which species are actually present in the bacterioplankton. One of the reasons is the lack of a universal medium for isolating marine bacteria

from natural environments. On traditional agar plates (e.g. ZoBell 2216 medium) one can isolate only a maximum of 0.7% of the microscopic direct counts of bacteria after acridine orange staining (Zimmermann et al. 1978, Brettar & Höfle 1993, Höfle & Brettar 1996). Most bacterial species from natural environments are difficult to identify with the phenotypic methods commonly used in clinical laboratories. For slow-growing and morphologically variable organisms, traditional phenotypic identification is time-consuming and very difficult. The identification of bacteria from environmental samples and the interpretation of results obtained exclusively by phenotypic methods are very often misleading (Stager & Davis 1992). A good alternative when phenotypic characterisation methods fail is the 16S ribosomal DNA-based identification of bacteria (Stackebrandt & Goebel 1994, Drancourt et al. 2000, Hagström et al. 2002).

The aim of our study was to isolate and identify several heterotrophic bacteria from the brackish waters of the southern Baltic Sea and to produce a morphological, biochemical, physiological and molecular description of them. This paper is the first report on the biochemical and molecular identification of heterotrophic bacteria from the brackish waters of the Gulf of Gdańsk and the Gdańsk Deep.

2. Material and methods

2.1. Sampling, isolation and growth conditions

The water samples were taken from the southern Baltic in 2003–2004 under sterile conditions using a Niskin PVC water sampler. Table 1 lists the sampling sites and isolation periods. The salinity at the sampling sites was about 6–7 PSU. After collection, the water was transported to the laboratory, where isolation procedures were immediately set in motion. The

Table 1. Origin of the bacterial strains

Strain number	Sampling site	Isolation date
1	Surface coastal water during diatom bloom, Sopot Pier	02.03.2004
2	Surface water from the Gdańsk Deep (54°50'N 19°19'E)	23.11.2003
3	Surface water from the Gulf of Gdańsk (54°30'N 19°06'E)	22.11.2003
4	Surface coastal water, Sopot Pier	22.10.2003
5	Surface coastal water during *Nodularia spumigena* bloom, Sopot Pier	05.08.2004
6	Surface water from the Gulf of Gdańsk (54°26'N 19°03'E)	23.03.2004

bacteria were isolated by serial dilution with sterile water in the 1–10^{-6} range. 100 μl of water from each dilution were spread onto Petri dishes containing ZoBell 2216 agar medium (Rheinheimer 1977). Following aerobic incubation at 30°C for 7 days single colonies of each bacterium were isolated. The purity of the cultures was confirmed by serial plating onto the same medium. The strains were stored at −80°C in ZoBell 2216 medium supplemented with 50% glycerol and were grown from this stock when needed.

2.2. Morphological, biochemical and physiological characteristics

The cell morphology of the isolated strains was examined by light microscopy using Gram staining of 24–48 h old cultures grown on ZoBell 2216 agar plates (Moaledj 1986).

The following physiological tests were carried out: catalase and oxidase reactions (Kędzia 1990); utilisation of D-glucose, D-mannitol, lactose, urea and ornithine; formation of gas from D-glucose; deamination of phenylalanine; reduction of nitrate; production of indole; decarboxylation of lysine (Kędzia 1990, Smibert & Krieg 1994). The tests were read after 24 h of incubation at 37°C. Physiological reactions of Gram-negative isolates were also tested using the ID 32 GN identification system for Gram-negative rods (bioMérieux) with automatic reading according to the manufacturer's instructions. For the Gram-positive coccus, sensitivity to the antibiotic furazolidone was checked.

2.3. Scanning electron microscopy (SEM) investigations

Cell size and form were determined by scanning electron microscopy. Cells were taken from the agar isolation medium and rinsed 3–5 times with 1 ml of 0.2 M potassium phosphate buffer. After the last rinsing, the cells were suspended in 4% glutaraldehyde and incubated for 2 hours at room temperature. The cells were then dehydrated in a series of increasing ethanol concentrations for 15 minutes each. Dehydrated cells were air-dried in an exsiccator, sputter-coated with 10–20 nm of Au and examined at 5 kV under a Hitachi S3200N scanning electron microscope.

2.4. DNA isolation; 16S rDNA gene amplification and sequencing

Bacterial cells were lysed using the freeze (in liquid nitrogen) and thaw (at 60°C) technique (five cycles). The primers used for 16S rDNA amplification – GM5F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTAGAGTTT-3') – have been described by Muyzer et al. (1995). Amplification was carried out by adding 10 μl of extracted

DNA to 40 μl of a PCR mixture containing 18.5 μl distilled water, 100 μg BSA (bovine serum albumin), 1× REDTaq PCR Buffer, 200 μM of each deoxynucleotide, 250 ng of each primer, and 2.5 U of REDTaq^{TM} DNA polymerase (Sigma). The reaction was performed in an Omni Gene Thermocycler (Hybaid UK) with an initial incubation of 4 minutes at 95°C, followed by 30 cycles of 1 minute at 95°C, 1 minute at 53°C, 1 minute at 72°C, and a final incubation for 10 minutes at 72°C. The amplified PCR products were electrophoresed and visualised with ethidium bromide using 2% agarose gel. Gel extraction of nucleic acids was carried out using a QIAquick Gel Extraction Kit (QIAGEN) and commercially sequenced. The nucleotide sequences were compared to sequences from the NCBI GenBank using the nucleotide-nucleotide BLASTN 2.2.10 method (Altschul et al. 1997).

3. Results

3.1. Phenotypic and physiological characteristics

All the isolated bacterial strains were aerobic and non-motile – five were Gram-negative, one was Gram-positive. Table 2 summarises the physiological and biochemical properties differentiating the isolates. Table 3 lists the results obtained using the ID 32 GN automatic identification

Table 2. Physiological characteristics of the isolated bacteria

Characteristic	Strain number					
	1	2	3	4	5	6
Gram staining	-	-	-	+	-	-
Catalase reaction	+	+	+	+	+	+
Oxidase reaction	-	+	-	-	+	+
Utilisation of D-glucose	+	+	+	-	-	-
Utilisation of D-mannitol	-	-	-	-	-	-
Utilisation of lactose	-	-	-	-	-	-
Utilisation of urea	-	-	-	+	-	+
Utilisation of ornithine	-	-	-	-	-	-
Formation of gas from D-glucose	-	-	-	-	-	-
Deamination of phenylalanine	-	-	-	-	-	-
Reduction of nitrate	-	-	-	-	-	-
Production of indole	-	-	-	-	-	-
Decarboxylation of lysine	-	-	-	-	-	-
Sensitivity to furazolidone	nd	nd	nd	resistant	nd	nd

+ positive reaction, - negative reaction, nd – not determined.

Table 3. Results from the automatic identification system for Gram-negative rods ID 32 GN

Substrates	Strain number				
	1	2	3	5	6
L-rhamnose	+	-	-	-	+
N-acetyl-glucosamine	-	-	-	-	-
D-ribose	-	-	-	-	+
Inositol	-	-	-	-	+
D-saccharose (sucrose)	-	-	-	-	+
D-maltose	-	+	-	+	+
Itaconic acid	-	-	?	-	+
Suberic acid	-	-	-	+	-
Sodium malonate	-	-	-	-	+
Sodium acetate	-	-	-	?	-
Lactic acid	-	-	+	?	+
L-alanine	-	-	-	-	?
Potassium 5-ketogluconate	-	-	-	-	+
Glycogen	+	-	+	+	+
3-hydroxybenzoic acid	-	-	-	-	-
L-serine	-	-	-	-	-
D-mannitol	-	-	-	?	-
D-glucose	-	-	-	-	+
Salicin	-	?	-	-	+
D-melibiose	-	-	-	+	-
L-fucose	-	-	-	-	-
D-sorbitol	-	-	-	-	-
L-arabinose	-	-	-	-	+
Propionic acid	-	-	-	?	+
Capric acid	-	-	-	-	+
Valeric acid	-	+	-	?	-
Trisodium citrate	-	-	-	-	+
L-histidine	-	-	-	-	+
Potassium 2-ketogluconate	-	-	-	+	+
3-hydroxybutyric acid	-	-	-	-	-
4-hydroxybenzoic acid	?	-	-	+	?
L-proline	-	-	-	+	+
Time of incubation [days]	14	3	8	3	8

+ positive reaction, - negative reaction, ? – ambiguous reaction.

system for Gram-negative rods (bioMérieux). Since the affiliations of the bacterial strains were very ambiguous, only the positive or negative reaction

of all the substrates on each strip is indicated. On the basis of an additional test, strain 4 proved to be resistant to the antibiotic furazolidone.

3.2. Morphology

Most of the strains studied were short rods, and one had a coccoid form. Like most marine bacteria, each microorganism formed coloured colonies. Table 4 summarises the morphology of the cells and their colonies, and Fig. 1 illustrates the cell size and form of the bacterial strains examined.

Table 4. Morphology of bacterial cells and their colonies isolated during this study

Strain number	Cell form	Cell length [μm]	Colony pigmentation	Colony size [mm]	Colony form
1	rod	3–4	white	1.5–5	non-convex, with shredded edges, in the middle much denser and white; outside nearly transparent
2	rod	1–2	pink	0.5–2	elongated, shiny, spilled on the agar, with regular edges
3	rod	0.5–1.5	orange	0.5–0.7	convex, shiny, with non-regular edges
4	coccus	0.5–1	yellow	1–3	circular, convex, shiny, with regular edges
5	rod	1–2	dark-yellow	0.5–2	circular, convex, shiny, with regular edges
6	rod	1–2	cream-coloured	0.1–1	circular, convex, shiny, with regular edges

3.3. 16S rDNA sequencing

Approximately 500 bp of 16S rDNA fragments were obtained for all the isolates examined. The 16S rDNA sequence similarity to that of a previously characterised bacterial species was $>98\%$ in five of the six strains, and 96% in the remaining strain (No 2). Table 5 summarises the identification of the isolates based on 16S rDNA sequences.

4. Discussion

Interest in and knowledge of marine heterotrophic bacterioplankton isolated from the Baltic Sea has increased in recent years (Höfle & Brettar 1996, Pinhassi et al. 1997, Brettar et al. 2002, 2003, 2004a,b, Salomon et al. 2003), mainly because of the introduction of 16S rDNA analysis – a useful

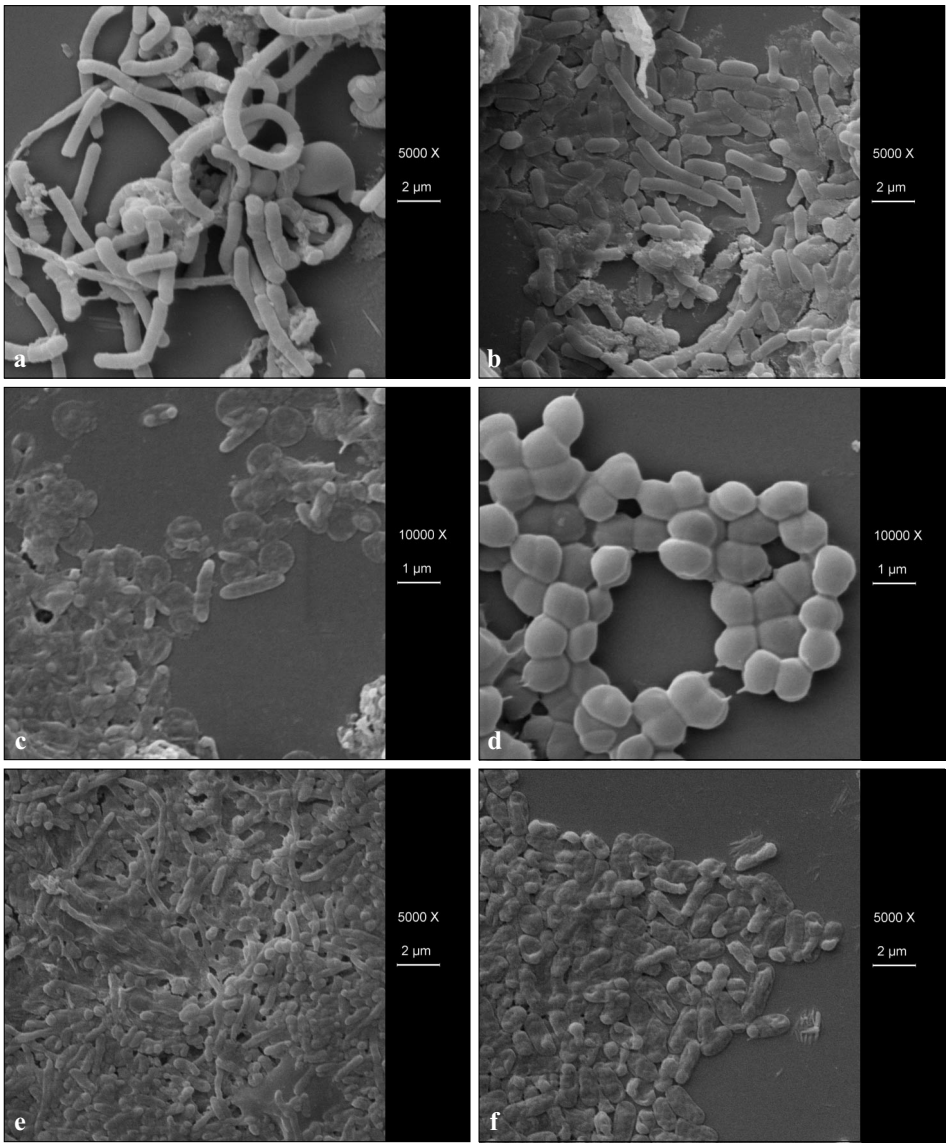

Fig. 1. Scanning electron micrographs of the heterotrophic bacteria studied; strain 1 (a), strain 2 (b), strain 3 (c), strain 4 (d), strain 5 (e) and strain 6 (f)

tool for identifying bacterial species and comparing their physiological, biochemical and morphological properties. Boivin-Jahns et al. (1995) emphatically demonstrated that the analysis of a small subunit of rDNA sequences is more efficient than the classical phenotypic methods for the identification of bacteria isolated from a natural environment. Nevertheless,

Table 5. 16S rDNA – based identification of bacterial isolates

Strain number	Sequence similarity [%]	Closest matching organism in GenBank (accession number)	References
1	100%	*Bacillus silvestris* (AJ006086)	(Rheims et al. 1999)
2	96%	*Pontibacter actiniarum* (AY989908)	(Nedeshkovskaya et al. 2005)
3	99%	*Erythrobacter vulgaris* strain 022 4–7 (AY706938)	(Ivanova et al. 2005)
		Erythrobacter vulgaris strain 022 2–12 (AY706937)	
		Erythrobacter vulgaris strain 022 2–10 (AY706935)	
		Erythrobacter sp. MB-16 (AF325446)	(Francis et al. 2001)
		Alpha proteobacterium MBIC2351 (AB012061)	(Hamada et al. unpublished)
4	99%	*Micrococcus luteus* (AF542073)	(Tang & Gillevet 2003)
5	99%	*Erythrobacter flavus* strain SW-52 (AF500005)	(Yoon et al. 2003)
		Erythrobacter flavus strain SW-46 (AF50004)	
		Erythrobacter citreus (AF118020)	(Vybiral et al. 1999)
6	98%	*Aurantimonas coralicida* strain WP1 (AY065627)	(Denner et al. 2003)
		Fulvimarina litoralis strain HTCC2156 (AY178863)	(Cho & Giovannoni 2003)

only a polyphasic study, analysing biochemical, physiological, morphological and molecular features, provides the best description of bacteria isolated from the marine environment.

We chose heterotrophic bacteria from the southern Baltic Sea for the following two reasons: they are a representative sample of bacteria from estuarine and brackish water environments; to date there have been no studies focusing on the identification of bacteria from the Gulf of Gdańsk and the Gdańsk Deep. The genera *Pseudomonas*, *Vibrio*, *Spirillum*, *Achromobacter*, *Flavobacterium* and *Bacillus* (in sediments) are very frequently present in sea water (ZoBell 1946). The Baltic Sea, containing as it does the largest volume of brackish water in the world, functions like a large estuary. So among our isolates there were some bacterial species not typical of the marine environment (e.g., *Micrococcus luteus*), especially as some of the water samples were taken from coastal waters.

In bacterial taxonomy it is commonly accepted that two bacteria do not belong to the same species unless the 16S rDNA sequence similarity is $<97\%$ (Stackebrandt & Goebel 1994, Hagström et al. 2000). In our experiments, the sequence of only one isolate (strain 2) displayed a 96% identity to the sequences available in the NCBI GenBank; for the other sequences the similarity was $>98\%$. The low sequence similarity of strain 2 suggests that it has not been isolated and described to date. But this hypothesis has still to be confirmed by further molecular and physiological studies.

Some studies have been done on bacteria isolated from the Baltic Sea, for example, the very interesting investigations by Bölter & Rheinheimer (1977, 1987). Having grouped the isolated bacteria by numerical analysis on the basis of 40 morphological and physiological features, these authors reported that *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Enterobacter* spp. and coryneforms are the most important taxa in the central Baltic. However, these genera are difficult to relate to any genus designated by molecular identification, because some of the microorganisms in that large collection of heterogeneous bacteria are nowadays classified in different genera or families. Some ten years later, Höfle & Brettar (1996) were unable to confirm these results. They isolated 123 bacterial strains from the Gotland Deep and determined their taxonomic position by high-resolution electrophoresis of low-molecular-weight RNA. More than 80% of the isolates were assigned to the families *Vibrionaceae*, *Enterobacteriaceae* and *Pseudomonadaceae*. Several studies have dealt with heterotrophic bacteria from the coastal regions of the Baltic Sea (Neilson 1980, Pinhassi et al. 1997). Neilson (1980) identified the isolates as *Alteromonas haloplanktis*, *Schewanella putrefaciens*, members of the family *Enterobacteriaceae*, and the genus *Pseudomonas*. Pinhassi et al. (1997), however, was able to confirm that seasonal dynamics affect the abundance of bacteria isolated from the northern Baltic Sea. The sequenced bacteria were placed among α- and γ-Proteobacteria and the cytophaga-flexibacter group. Our data confirm these results: in the course of our studies in the southern Baltic Sea we also found species belonging to the α subdivision of the class *Proteobacteria* (strains 3, 5 and 6).

Some new marine bacteria – *Idiomarina baltica*, *Belliella baltica*, *Rheinheimera baltica* and *Aquiflexum balticum* – have been isolated from the surface water of the central Baltic Sea (Brettar et al. 2002, 2003, 2004a,b).

Strain 1

16S rDNA analysis clearly demonstrated that with its 100% 16S rDNA sequence similarity to *Bacillus silvestris* (Rheims et al. 1999) strain 1

is a member of the genus *Bacillus*, which is particularly common in marine sediments. Comparison of physiological properties revealed some slight differences with respect to *B. silvestris*. Firstly, our isolate 1 was stained as a Gram-negative rod, a rare occurrence among aerobic rod-shaped bacteria (Kędzia 1990), since the cell wall structure of the bacilli was consistent with that of Gram-positive bacteria. When entering the stationary phase of growth, however, many sporeformes rapidly become Gram-negative. Secondly, strain 1 utilised L-rhamnose and glycogen but not D-ribose, whereas according to the results of Rheims et al. (1999) the opposite reactions took place. The sequence similarity between strain 1 and *B. silvestris* was 100%, which clearly indicates the same species affiliation.

Strain 2

The 16S rDNA sequence of strain 2 shared the highest degree of similarity with that of *Pontibacter actiniarum*, a new member of the phylum *Bacteroidetes*, pink-coloured bacteria associated with marine coelenterates (Nedeshkovskaya et al. 2005). However, an only 96% 16S rDNA sequence identity is too little to affiliate this isolate to any species existing in the GenBank. The majority of *Bacteroidetes* bacteria have been isolated from sea water, sediments or algae (Bowman et al. 2003, Brettar et al. 2004a,b, Yi & Chun 2004). The genus *Pontibacter* has rod-shaped cells, is motile by means of gliding, is Gram-negative, and does not form endospores. It is aerobic, a chemo-organotroph, and oxidase-, catalase- and alkaline phosphatase-positive. Our strain 2 exhibited the same characteristics, even though the oxidase reaction was weak and positive in long-term reactions. The main differences between isolate 2 and *P. actiniarum* described by Nedeshkovskaya et al. (2005) were the utilisation of D-glucose and D-maltose. On the basis of the ID 32 GN automatic identification system an affiliation to *Empedobacter brevis* with 68% probability was obtained after three days' incubation. This species also belongs to the phylum *Bacteroidetes*, but to the family *Flavobacteriaceae*, not the *Flexibacteriaceae* like *P. actiniarum*. Our conclusion is that neither the results of 16S rDNA analysis nor the physiological tests were able to provide an unequivocal identification of the strain. It is possible that our strain number 2 represents a new species. This hypothesis will, however, have to be confirmed in further studies, which will include the sequencing of further, possibly functional genes and detailed physiological examination.

Strain 3

Comparison of the 16S rDNA sequences indicated very close similarity ($>99\%$, differing by only 3 nucleotides) to four strains belonging to the genus

Erythrobacter – *Erythrobacter vulgaris* strain 022 4–7, 022 2–12, 022 2–10 and *Erythrobacter* sp. MB-16 – and to the Alpha proteobacterium MBIC 2351. The genus *Erythrobacter* was created in 1982 by Shiba & Simidu (1982) to accommodate marine species of aerobic anoxygenic phototrophs containing bacteriochlorophyll *a* (BChl *a*). *E. vulgaris* strains 022 4–7, 2–12, 2–10 were isolated from the starfish *Stellaster equestris* and soft coral collected in the South China Sea (Ivanova et al. 2005), and *Erythrobacter* sp. MB-16 from the sediments of Mission Bay (Francis et al. 2001). Our strain 3 had some of the phenotypic and physiological characteristics of bacteria from the genus *Erythrobacter*, for example, a Gram-negative cell wall, rod-shaped cells, orange colony pigmentation, and a catalase-positive reaction. Unfortunately, the oxidase reaction was negative, whereas an oxidase-positive reaction is typical of *Erythrobacter*. This genus has also been isolated from various marine environments (coastal waters, water collected on a beach, and open-ocean waters) in the Atlantic, Pacific and Indian Oceans. Sea surface temperatures in the sampling locations ranged from 8°C to 23°C (Koblížek et al. 2003). Our studies are in agreement with those of Pinhassi et al. (2003), who also found *Erythrobacter* in North Sea water. Strain 3 was isolated in November 2003 after a severe autumn storm that caused large amounts of North Sea water to flow into the Baltic.

Strain 4

The Gram-positive strain 4 was a catalase-positive coccus, often arranged in tetrads. It grew in circular, entire, convex, yellow-pigmented colonies approximately 3 mm in diameter. This bacterium was isolated from coastal water, which suggests that it may be terrigenous (sandy beach). The physiological and biochemical properties as well as the 16S rDNA sequence similarity (99%) of our isolate 4 clearly demonstrated that it belongs to the genus *Micrococcus*, with the highest similarity to the *M. luteus* biovar III isolated from an activated-sludge plant, which is yellow in colour, urease-positive, and does not utilise mannitol or phenylalanine (Wieser et al. 2002). The genus *Micrococcus* is highly cosmopolitan and may also occur in sea water, especially in water of such low salinity. The sensitivity test to the antibiotic furazolidone was used to distinguish *Micrococcus* sp. from *Staphylococcus* sp.

Strain 5

16S rDNA sequence analysis showed that strain 5 belongs to the genus *Erythrobacter,* with the closest similarity to a variety of strains belonging to this genus, such as *Erythrobacter flavus* or *Erythrobacter citreus*. Our strain 5 exhibited several physiological and biochemical features typical of the

Erythrobacter genus: yellow pigmentation, catalase- and oxidase-positive reactions (Shiba & Simidu 1982). Like *E. flavus* from the East Sea in Korea, it did not reduce nitrate to nitrite, or utilise D-glucose and maltose (Yoon et al. 2003). In comparison to *E. citreus*, a strain isolated from the western Mediterranean Sea (Denner et al. 2002), our isolate 5 did not utilise D-glucose, nor did it reduce nitrate to nitrite. Strain number 5 was therefore taken as resembling *E. flavus* rather than *E. citreus*, which has also been reported from the North Sea (Pinhassi et al. 2003).

Strain 6

16S rDNA analysis showed that strain 6 belongs to the class *Alphaproteobacteria*, with a 98% similarity to two species: *Aurantimonas coralicida* strain WP1 and *Fulvimarina litoralis* strain HTCC 2156. The *Aurantimonadaceae* is a small family of marine bacteria. To date there are two known and described species from this family: *Aurantimonas coralicida* (Denner et al. 2003), causing white plague in corals, which progressively destroys their tissues, and *Fulvimarina pelagi* (Cho & Giovannoni 2003), isolated from the water of the western Sargasso Sea. Both species showed positive reactions in oxidase and catalase tests. *F. pelagi* has also been noted in the Atlantic Ocean. On the basis of the ID 32 GN identification system for Gram-negative rods, we obtained only a very low resemblance to the species *Flavimonas oryzihabitans*, which is a yellow-pigmented, Gram-negative, oxidase-negative bacterium, isolated from damp environments, such as a rice paddies and sink drains (Kodama et al. 1985). However, this result did not correspond with the results of the 16S rDNA sequencing, which showed a 98% similarity to the sequences of the *A. coralicida* strain WP1 and the *F. litoralis* strain HTCC 2156, because both *A. coralicida* and *F. litoralis* belong to the class *Alphaproteobacteria*, whereas *F. oryzihabitans* belongs to the *Gammaproteobacteria*.

The isolated and sequenced bacteria belonged to a wide spectrum of different taxonomic groups of bacteria, namely, the families *Bacillaceae* (strain 1), *Flexibacteriaceae* (strain 2), *Sphingomonadaceae* (strains 3, 5), *Micrococcaceae* (strain 4) and *Aurantimonadaceae* (strain 6). Among our isolates only the *Cytophaga-Flavobacterium-Bacteroides* group had previously been reported in the surface water of the central Baltic Sea (Brettar et al. 2004a,b). Pinhassi et al. (2003) isolated 28 bacterial species from the Skagerrak-Kattegat front, and among these isolates was *Erythrobacter citreus*, from the family *Sphingomonadaceae*. The Skagerrak-Kattegat front is a meeting point between North Sea and Baltic Sea water. *Erythrobacter* sp. has also been noted in North Sea water.

Our results obtained from the isolation and identification of bacteria from the brackish water of the southern Baltic Sea suggest that typical marine bacteria as well as bacteria inhabiting other environments (fresh waters, sediments, soil) can occur in this environment. Whether they do in fact occur there, however, probably depends on their different tolerance to salinity.

The results of our studies are a clear indication that only biochemical and physiological tests in conjunction with 16S rDNA analysis can yield a realistic picture of the evolutionary relationships of diverse groups of bacteria. It was not possible to ascertain the true phylogeny expressing the diversification of bacteria by the traditional, phenotypic taxonomic methods of the late nineteenth and early twentieth centuries. Bacterial taxonomy and identification require molecular methods in addition to phenotypic data, but molecular methods cannot realistically be the first or only appropriate method.

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Appendix

Nucleotide sequence data of strain 1

GAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGT
GAAGAAGGATTTCGGTTCGTAAAACTCTGTTGCAAGGGAAGAACAAGTAGCGTAGTAACTGG
CGCTACCTTGACGGTACCTTGTTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTCCTTA
AGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGT
GCAGAAGAGGATAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACAC
CAGTGGCGAAGGCGACTGTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGGGGGTTT
CCGCCCCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGAC
TGAAACTCAAAGGA

Nucleotide sequence data of strain 2

GGAGGCAGCAGTAGGGAATATTGGGCAATGGCCGATAGGCTGACCCAGCCATGCCGCGTGCA
GGAAGAAGGCCTTCTGGGTTGTAAACTGCTTTTACCAAGGAAGAAAACGCCCATGCGTGGGT
AACTGACGGTACTTGGTGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG
AGGGTGCAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCCCGTTAAGTCA
GCGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCGTTGATACTGGCGGGCTTGAGTTCGGT
AAAGGCGGGCGGAACTGGCGGTGTAGCGGTGAAATGCTTAGATACCGCCAAGAACCCCGATT
GCGTAGGCAGCTCGCTGTGCCGAAACTGACGCTGAGGCACGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATGACTCGATGTTGGCGATACACAGTC
AGCGTCCAAGCGAAAGCGTTAAGTCATCCACCTGGGGAGTACGCCCGCAAGGGTGAAACTCA
AAGGAAT

Nucleotide sequence data of strain 3

GGGGAATATTGGACAATGGCCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCT
TAGGCTTGTAAAGCTCTTTTACCAGGGATGATAATGACAGTACCTGGAGAATAAGCTCCGGC
TAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGAGCTAGCGTTGTTCGGAATTACTGGGC
GTAAAGCGCGCGTAGGCGGCTTTTCAAGTCAGGGGTGAAATCCCGGGGCTCAATCCCCGGAA
CTGCCCTTGAAACTGGATGGCTAGAATACTGGAGAGGTGAGTGGAATTCCGAGTGTAGAGGT
GAAATTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACTCACTGGACAGTTATTGAC
GCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGATAACTAGCTGCTTGGGCTCATGGAGCTTGGGTGGCGCAGCTAACGCA

Nucleotide sequence data of strain 4

AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGG
ATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGC
AGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGAGCG

TTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGTCGTGAAAGTC
CGGGGCTTAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGG
AATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGT
CTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGACCATTCCACGGTTTCCGCGCCG
CAGCTAACGCATTAAGTGCCCCG

Nucleotide sequence data of strain 5

CCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAT
GAAGGCCTTAGGGTTGTAAAGCTCTTTTACCAGGGATGATAATGACAGTACCTGGAGAATAA
GCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGAGCTAGCGTTGTTCGGAAT
TACTTGGGCGTAAAGCGCACGTAGGCGGCTTTTCAAGTCAGGGGTGAAATCCCGGGGCTCAA
CCCCGGAACTGCCCTTGAAACTGGATGGCTAGAATACTGGAGAGGTGAGTGGAATTCCGAGT
GTAGAGGTGAAATTCGTAGCATATTCGGAAGAACACCAGTGGCGAAGGCGACTCACTGGACA
GTTATTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAACCTGGTAGTCCA
CGCCGTAAACGATGATAACTAGCTG

Nucleotide sequence data of strain 6

GAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGT
GATGAAGGCCCTAGGGTTGTAAAGCTCTTTCAGTGGGGACGATAATGACGGTACCCACAGAA
GAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGG
AATTACTGGGCGTAAAGCGCACGTAGGCGGACTTTTAAGTCAGGGGTGAAATCCCGGGGCTC
AACCCCGGAACTGCCTCTGATACTGGAAGTCTCGAGTGTGGTAGAGGTGAGTGGAATTGCGA
GTGTAGAGGTGTAAATTCGTAGATATTCGGCAGGAACACCAGTGGCGAAAGGCGGGGTCACT
GG