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Rheinheimera baltica gen. nov., sp. nov., a blue-coloured bacterium isolated from the central Baltic Sea

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A set of taxonomically unique, blue-coloured bacterial isolates are described on the basis of physiological and biochemical characterization, fatty acid profiling and analyses of 16S rDNA sequences. The flagellated, non-fermentative strains were isolated in 1986, 1987 and 1998 from different layers of the water column of the central Baltic Sea. According to comparison of the 16S rDNA sequences, all strains are very closely related to each other and to strains from several other marine environments, including the deep sea. Thus, the described species seems to be widespread in marine habitats. According to DNA–DNA hybridization, the strains described can be considered to belong to the same species. The bacteria grew at temperatures from 4 to 30 °C, with an optimum around 20–25 °C. Growth was observed at salinities from 0 to 30, with an optimum between 10 and 30 and no growth at high salinities. The dominant fatty acids were 16:1ω7*c*, 16:0 and 18:1ω7*c*. The G+C content of the DNA ranged from 47·8 to 48·9 mol%. Phylogenetic analyses of the 16S rDNA sequences revealed a clear affiliation with members of the γ-*Proteobacteria*. The closest relationship was seen with *Alishewanella fetalis* but, in terms of physiology, colour and fatty acids, the bacteria described are rather distant from *A. fetalis*. To honour the marine microbiologist Gerhard Rheinheimer, the name *Rheinheimera baltica* gen. nov., sp. nov., is suggested for the Baltic isolates, with the type strain OSBAC1^T (= DSM 14885^T = LMG 21511^T).

Keywords: marine bacteria, Baltic Sea, '*Curacaobacter*'

INTRODUCTION

In summer 1986, we isolated intense blue-coloured colonies from different depths of the water column of the Gotland Deep, an anoxic basin in the central part of the Baltic Sea. Analysis of these bacterial strains using low-molecular-weight (LMW) RNA fingerprinting revealed a unique profile, classified as genotype B. This LMW RNA profile was first shown for the strain OS140 in a study of bacterial isolates obtained from the Gotland Deep in 1986 (Höfle & Brettar, 1996). In September 1998, we had the opportunity to screen bacterial isolates obtained from different depths and stations in the central Baltic Sea for blue-coloured bacteria. We succeeded in obtaining a final set of 11 blue-coloured isolates of genotype B, the taxonomic position of which was confirmed by LMW RNA fingerprinting and 16S rDNA sequence analysis.

Based on 16S rDNA sequence comparison, the 11 blue-coloured isolates originating from the water column of the central Baltic turned out to be closely related to isolates from other marine sites including deep-sea environments (Takami *et al.* 1999) and to clones from the central Baltic Sea. These blue-coloured bacteria are phylogenetically very distant from other described marine bacteria. They are most closely related to a clinical isolate, recently described as *Alishewanella fetalis* (94·8% similarity according to 16S rRNA comparison), isolated from a human foetus

A phylogenetic tree based on 16S rRNA gene sequences including a wider selection of relatives of *Rheinheimera baltica* gen. nov., sp. nov., and details of the fatty acid composition of four of the novel strains are available in IJSEM Online (http://ijs.sgmjournals.org/).

Abbreviation: TMAO, trimethylamine *N*-oxide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains OS140, OSBAC1^T, OS550, OSBAC5 and OSBA33 are AJ002006 and AJ441080–AJ441083.

(Fonnesbech Vogel *et al*., 2000). As the blue Baltic Sea isolates seem to represent a novel and important group of marine bacteria, five representative isolates were studied further with respect to their physiology, fatty acids, LMW RNA profiles, G+C content and 16S rDNA sequence analysis.

METHODS

Bacterial strains, isolation and growth conditions. During summer cruises on board RV *Poseidon* and RV *Aranda* in 1986, 1987 and 1998 in the central Baltic Sea, 11 blue-coloured bacterial strains were isolated from two different stations, the Gotland Deep (BY15: 57·1920° N, 20·0302° E) and a station at the entrance to the Gulf of Finland (LL12: 59·2900° N, 22·5398° E). The strains were isolated from the surface water of both stations and from deeper layers, down to 180 m, from the Gotland Deep. Eight isolates were obtained from the oxic part of the water column and three from the anoxic part of the Gotland Deep, i.e. below 130 m. Details of environmental conditions and sampling procedures are given elsewhere (Brettar & Rheinheimer, 1991, 1992; Rheinheimer, 1992; Brettar & Höfle, 1993). Data on the origin of the five isolates OS550, OS140, OSBAC1^T, OSBAC5 and OSBA33, characterized further in this study, are given in Table 1. Media for isolation were ZoBell agar (Oppenheimer & ZoBell, 1952) and nutrient broth plus nitrate (2 g KNO_3 l^{-1}). Details of the isolation procedures are given by Höfle & Brettar (1995, 1996). Strains grew well on ZoBell agar and half-strength as well as full-strength Marine broth or agar (Difco).

Physiological, biochemical tests and morphology. Unless indicated otherwise, the phenotypic properties of five of the novel strains were determined by using standard procedures (Gerhardt *et al*., 1994; Stanier *et al*., 1966). A number of key characteristics such as the Gram reaction, cell shape [phase-contrast microscopy, electron microscopy after negative staining with 1·25% (w/v) uranyl acetate], cytochrome oxidase and catalase were determined. Furthermore, production of hydrogen sulphide (Dye, 1968), aminopeptidase (Merck Bactident test), haemolysis, acid production from glucose, ribose and arabinose and hydrolysis of starch, gelatin, Tween 80 and lecithin were tested. Strains were additionally characterized by the whole test spectrum of the identification systems API 50CH, API 20NE and API ZYM (bioMérieux) at 20 °C. Growth at different temperatures was tested at 4, 10, 20, 25, 30 and 37 °C. Growth at different salinities was tested at 0, 1, 3, 6 and 10% (w/v) NaCl. For these tests, we used half-strength Marine broth (Difco), except for the salinity test, where half-strength Caso medium (DSMZ Catalogue no. 220) was supplemented with the appropriate amount of NaCl.

To test for anaerobic respiration, strains were inoculated in Marine broth (Difco) plus the electron acceptors at a final concentration of 10 mmol l^{-1}. Incubation was done anaerobically in the dark for up to 18 days at 20 °C. No growth was observed in Marine broth in an anoxic environment without addition of electron acceptors. Growth under anoxic conditions in presence of the added electron acceptors was considered as an indicator of electron acceptor utilization. As a positive control, *Shewanella baltica* OS155, a Baltic Sea strain able to use the electron acceptors provided (Ziemke *et al*., 1998), was used for a comparison.

Phylogenetic analysis based on 16S rRNA gene sequences. Genomic DNA was prepared from individual colonies as described by Moore *et al*. (1996). 16S rRNA genes were amplified by PCR (Mullis & Faloona, 1987) and the PCR products were sequenced directly as described previously (Moore *et al*., 1999).

The 16S rDNA sequence of the novel isolates was aligned automatically and then manually by reference to a database of 35000 already-aligned bacterial 16S rDNA sequences. The same sequence was then used in a BLAST search against the current content of the EMBL database (*Bacteria* division) to check for the presence of newly submitted related sequences. Phylogenetic trees were constructed according to three different methods (bioNJ, maximum-likelihood and maximum-parsimony). For the neighbour-joining (NJ) analysis, matrix distances were calculated according to Kimura's two-parameter correction. Bootstraps were done using 500 replications, bioNJ and Kimura's two-parameter correction. BioNJ analysis was done according to Gascuel (1997) and maximum-likelihood and maximum-parsimony data were from PHYLIP (Phylogeny Inference Package, version 3.573c; distributed by J. Felsenstein, Department of Genetics, UW, Seattle, WA, USA). Phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996) and Claris Draw software for Apple Macintosh. Domains used to construct phylogenetic trees were regions of the small-subunit rDNA sequences available for all sequences and excluding positions likely to show homoplasy.

For the tree shown in Fig. 2, only those sequences of related genera, mostly from type strains (31 sequences), for which almost the entire 16S rDNA sequence, corresponding to positions 79–1427 of the sequence of the novel isolates, was included in the analysis were retained. The topology shown is that of the bootstrap analysis, as it has been demonstrated that this topology is often better than that of a simple NJ analysis (Berry & Gascuel, 1996); for further consideration of the treeing analysis, see Ivanova *et al*. (2002). The analysis was done with all 31 sequences, but the tree shown is a subset of the total analysis (the full tree is available as supplementary material in IJSEM Online at http://ijs.sgmjournals.org/).

DNA isolation and spectrophotometric DNA–DNA hybridization. DNA was isolated by chromatography on hydroxyapatite (Cashion *et al*., 1977). DNA–DNA hybridization was carried out as described by De Ley *et al*. (1970) with the modifications described by Huß *et al*. (1983) and Escara & Hutton (1980) using a Gilford System model 2600 spectrometer. Renaturation rates were calculated with the program TRANSFER.BAS (Jahnke, 1992).

Determination of the G+C content. The DNA G+C content of the strains was determined by HPLC analysis of hydrolysed DNA according to Tamaoka & Komagata (1984) and Mesbah *et al*. (1989).

Cellular fatty acid profiles. The strains were grown on half-strength Marine broth (Difco) for 24 h at 28 °C. Fatty acid methyl esters were obtained from washed cells by saponification, methylation and extraction. Analysis by GC was controlled by MIS software (Microbial ID) and peaks were automatically integrated and identified by the Microbial Identification software package (Sasser, 1990).

RESULTS AND DISCUSSION

Physiological, morphological and biochemical characteristics

The novel strains are Gram-negative rods or cocci, 0·5–1·5 μm wide and 0·9–4·5 μm long, with a polar

Table 1. Phenotypic characterization of the Baltic Sea strains in comparison with *A. fetalis*

Data for *A. fetalis* were taken from Fonnesbech Vogel *et al.* (2000). Characters are scored as: +, good response/growth; ++, very good response/growth; −, no response/growth; w, weak response/growth. All strains shown stain Gram negative, show good growth at 30 °C and are positive for the Gram string test, catalase, cytochrome oxidase and β-glucosidase (aesculin). All strains are negative for acid production from D-glucose, D-ribose and D-arabinose, haemolysis, growth in 10 % NaCl and reduction of ferric iron. All five Baltic Sea strains are positive for hydrolysis of starch, gelatin, Tween 80 and lecithin, aminopeptidase activity, growth at pH 5·7 in Marine broth and API tests for alkaline phosphatase, leucine arylamidase, trypsin and naphthol phosphohydrolase. All tests of the API 50CH, API 20NE and API ZYM kits that are not listed gave negative results.

Character	Baltic Sea strains					*A. fetalis* CCUG 30811^T
	OS550	OS140	OSBAC1^T	OSBAC5	OSBA33	
Isolation:						
Site of isolation	BY15	BY15	LL12	BY15	BY15	
Depth (m)	180	10	5	50	50	
Date of isolation	Aug. 1987	Aug. 1986	Sep. 1998	Sep. 1998	Sep. 1998	
Cell shape	Rod	Rod	Rod/coccus	Rod/coccus	Rod/coccus	Rod
Flagella	+	+	+	+	+	−
DNA G+C content (mol %)	48·4	47·8	48·9	ND	ND	50·6
Growth at/in:						
0 % NaCl	+	+	+	+	+	ND
1 % NaCl	++	++	++	++	++	ND
3 % NaCl	++	++	+	+	+	ND
6 % NaCl	+	−	−	−	−	+
4 °C	+	w	+	+	+	−
10 °C	+	+	+	+	+	ND
20 °C	++	+	++	++	++	ND
25 °C	++	+	++	++	++	w
37 °C	−	−	−	−	−	+
Reduction of:						
Thiosulphate	−	−	−	−	−	+
Sulphite	−	−	−	−	−	+
Nitrate	−	−	−	−	−	+
TMAO	−	−	−	−	−	+
Utilization of (API):						
D-Glucose	+	−	+	+	+	ND
N-Acetyl glucosamine	+	−	+	+	+	ND
Aesculin	+	−	−	−	−	ND
Cellobiose	+	−	+	+	+	ND
Maltose	+	−	+	+	+	ND
Sucrose	+	−	w	w	+	ND
Starch	+	−	+	+	+	ND
Glycogen	+	−	+	+	+	ND
β-Gentiobiose	+	−	+	+	+	ND
Assimilation of (API):						
Glucose	++	−	++	+	+	−
N-Acetyl glucosamine	+	−	++	+	+	−
Maltose	++	−	++	+	++	−
Enzymic activity (API):						
Protease (gelatin)	++	+	++	+	++	+

flagellum (Fig. 1). Some strains were pleomorphic, i.e. rods and cocci of variable size occurred at the same time (Table 1). Colonies are circular, smooth, convex, entire and opaque with a blue colour. The appearance of colonies can range from colonies that are stained completely dark blue to violet to colonies with light-blue centres and transparent margins.

In terms of physiological features (Table 1), all five strains analysed showed a rather homogeneous pat-

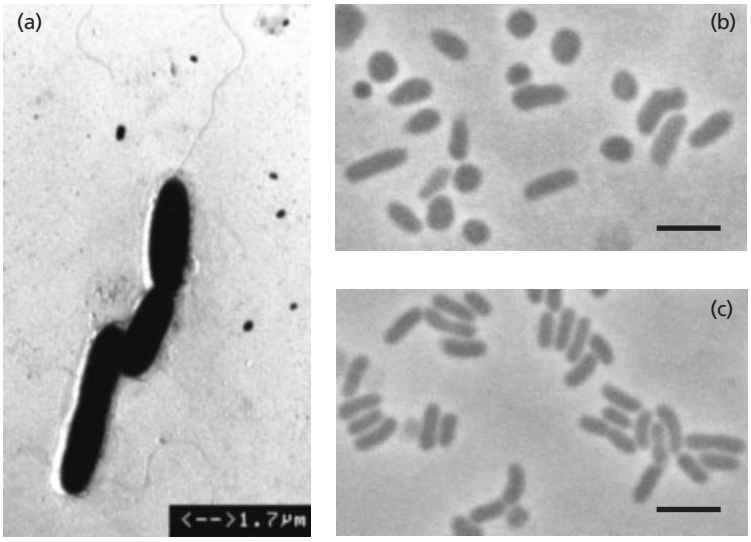

Fig. 1. (a)–(b) Cells of *Rheinheimera baltica* gen. nov., sp. nov. OSBAC1^T after negative staining with uranyl acetate visualized by electron microscopy (a) and phase-contrast microscopy (b; bar, 5 μm). (c) Cells of strain OS550 visualized by phase-contrast microscopy. Bar, 5 μm. Cells shown were grown in half-strength Marine broth (Difco) to the exponential growth phase.

tern. The strains were catalase- and cytochrome oxidase-positive. Growth was observed at temperatures from 4 to 30 °C, with an optimum around 20–25 °C. Good growth was observed at salinities from 0 to 3 %, with an optimum at 1–3 %. All strains were able to grow in Marine broth under oxic conditions. No growth occurred under anoxic conditions, even in the presence of a set of different potential electron acceptors [thiosulphate, sulphite, nitrate, trimethylamine *N*-oxide (TMAO), ferric iron]. Under the same conditions, growth of *Shewanella baltica* OS155, which is able to use the above-mentioned electron acceptors, was observed. Therefore, we conclude that none of the electron acceptors provided can be utilized by the blue-coloured strains.

The novel strains showed positive results for many of the API 50CH, API NE and API ZYM tests (Table 1). Results were comparable for the tested strains, except for substrate utilization by strain OS140. Electron microscope analysis revealed that strain OS140 contained phages that occasionally caused cell lysis when the strain was growing in liquid culture, which might have caused the negative results.

Comparison of the physiological features of the Baltic strains with those of *A. fetalis* is restricted because of a lack of physiological data for this species (Table 1). Cells of the novel strains were flagellated, whereas cells of *A. fetalis* are not. In terms of physiology, major differences were observed for the temperature range. *A. fetalis* grows best at 37 °C, whereas no growth of the novel strains occurs at this temperature. Salinity of 6 % is tolerated well during growth of *A. fetalis*, but only one of the five novel strains tested showed growth under these conditions. *A. fetalis* did not show any substrate assimilation with API 20NE, while the novel strains assimilated glucose, *N*-acetyl glucosamine and maltose. A major difference was the utilization of electron acceptors. While the novel blue-coloured strains were not able to utilize any of the electron acceptors for growth under anoxic conditions, *A. fetalis* used thiosulphate, TMAO and nitrate (Fonnesbech Vogel *et al.*, 2000).

The DNA G+C content of three strains analysed ranged from 47·8 to 48·9 mol %. The G+C content of the *A. fetalis* type strain was 50·6 mol %. Distinction between the novel strains and *A. fetalis* cannot be done on the basis of these G+C contents because the difference is too small (Stackebrandt & Liesack, 1994).

LMW RNA profiles of the novel strains

The novel strains showed a distinct LMW RNA pattern that allowed them to be differentiated from other Baltic Sea isolates (profile of genotype B; Höfle, 1990, 1998; Höfle & Brettar, 1996). The 11 strains isolated between 1986 and 1998 were recognized by their unique LMW RNA fingerprint among other isolates of similar appearance. The abundance of strains of the novel taxon among the isolates in 1986 was four of a total of 123 strains, i.e. 3 % of the isolates.

Genotypic relationships within the tested strains

The four strains subjected to DNA–DNA hybridization showed high similarity, ranging from 87·1 % between OS550 and OSBAC5 to 99·2 % between OSBAC1^T and OSBAC5 (Table 2). This high level of similarity was observed for strains that were isolated over 12 years from two different stations in the central Baltic Sea. Strains OSBAC1^T and OSBAC5, with the high similarity of 99·2 %, were even isolated from different stations in September 1998, at a distance of about 300 km, and from two different depths, 5 and 50 m. Comparison of the 16S rDNA among the set of novel strains showed similarities of at least 98·7 % when nearly complete 16S rDNA sequences were compared (Table 2).

Table 2. DNA base composition, DNA–DNA relatedness and 16S rDNA similarity for the Baltic Sea strains and related strains

Strain	Origin	G+C content (mol %)	DNA–DNA similarity (%) with:			16S rRNA accession no.	16S rDNA sequence similarity to OSBAC1^T (%)		Reference
			OS550	OS140	OSBAC1^T		Complete*	Partial†	
OS140	Baltic Sea, 1986	47·8	93·1			AJ002006	98·7	96·5	This study
OSBAC1^T	Baltic Sea, 1998	48·9	91·8	97·2		AJ441080	(100)		This study
OSBAC5	Baltic Sea, 1998	ND	87·6	97·7	99·2	AJ441082	98·7	96·5	This study
OS550	Baltic Sea, 1987	48·4	ND	ND	ND	AJ441081	100 (999)		This study
OSBA33	Baltic Sea, 1998	ND	ND	ND	ND	AJ441083		96·5	This study
MC-6	Marine isolate	ND	ND	ND	ND	AF186703	100 (889)		Alavi *et al.* (2001)
HTB021	Pacific, deep-sea mud	ND	ND	ND	ND	AB010859	96·5		Takami *et al.* (1999)
HTB010	Pacific, deep-sea mud	ND	ND	ND	ND	AB010858	96·6		Takami *et al.* (1999)
HTB019	Pacific, deep-sea mud	ND	ND	ND	ND	AB010860	96·8		Takami *et al.* (1999)
A. fetalis CCUG 30811^T	Clinical isolate	50·6	ND	ND	ND	AF144407	94·8		Fonnesbech Vogel *et al.* (2000)

* Numbers of nucleotides aligned with OSBAC1^T less than 1300 nt are given in parentheses.

† Positions 28–490 according to the *E. coli* numbering.

ND, No data.

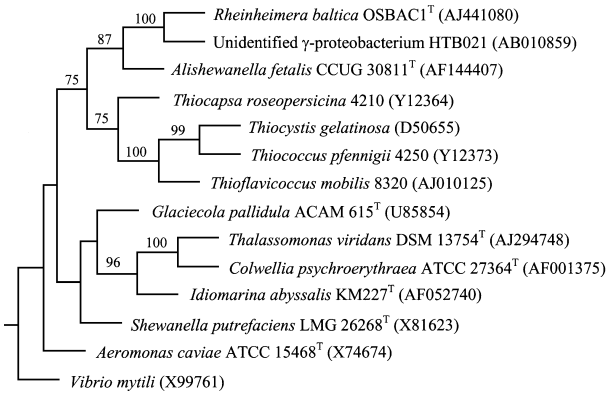

Fig. 2. Phylogenetic tree of a subset of the *γ-Proteobacteria* based on 16S rDNA sequence comparison. The topology shown is a subset, restricted to strains related most closely to the novel isolates, of a larger analysis that included most type species that were close neighbours of the novel sequence from *Rheinheimera baltica* OSBAC1^T (the full tree is available in IJSEM Online at http://ijs.sgmjournals.org/). The tree was obtained using the bioNJ algorithm and 500 bootstrap replications with Kimura's two-parameter correction for the distances. Bootstrap percentages are indicated only for branches that were also retrieved by maximum-parsimony and maximum-likelihood ($P < 0{\cdot}01$); these branches only should be considered to identify robust clusters in this analysis.

Analysis of the phylogenetic position based on 16S rRNA gene sequence comparison

16S rDNA sequence analyses (Fig. 2) revealed that the bacteria studied are members of the *γ-Proteobacteria* and that they clustered robustly with the species *A. fetalis*. A very narrow clustering was also obtained with strains HTB010 and HTB019, isolated from deep-sea mud (Takami *et al.*, 1999), and strain MC-6, isolated from *Pfiesteria*-like dinoflagellate cultures (Alavi *et al.*, 2001). Comparison of 16S rDNA sequences, based on nearly complete sequences, with the closely related Japanese deep-sea strains (HTB010, HTB019, HTB021) gave similarities of 96·5–96·8 %. With *A. fetalis*, the similarity was 94·8 % (Table 2).

Cellular fatty acid profiles

The fatty acid profiles of four strains analysed showed rather similar patterns (fatty acid profiles are available as supplementary material in IJSEM Online at http://ijs/sgmjournals/org/). The similarity was most pronounced for strain OSBAC1^T in comparison to strain OSBAC5, which coincides with the highest DNA–DNA homology (99·2 %) observed. The dominant fatty acids were 16:1ω7*c* (mean ± SD of 34·8 ± 4·8 % for the four strains), 16:0 (22·1 ± 3·6 %) and 18:1ω7*c* (15·2 ± 5·0 %). For the closest relative, *A. fetalis* CCUG 30811^T, the dominant fatty acids were 17:1ω8*c* (19·5 %), 16:1ω7*c* (19·0 %) and 17:0 (10·3 %) (Fonnesbech Vogel *et al.*, 2000), a rather different spectrum of abundant fatty acids in comparison with the Baltic

Sea strains. However, the temperature and the medium applied during growth of *A. fetalis* were different from our growth conditions. Therefore, the comparability of the fatty acid data is limited.

Conclusion

Of validly described species, the Baltic Sea strains OSBAC1^T, OSBAC5, OS550, OS140 and OSBA33 are phylogenetically most closely related to the clinical isolate *A. fetalis*. 16S rDNA gene sequence comparison shows that there are a number of bacteria isolated from marine environments that are related more closely than *A. fetalis*. Besides the phylogenetic relationship, all other features are different for the marine bacteria. The novel blue-coloured strains are flagellated, have a different temperature range and salt tolerance for growth and utilize and assimilate different spectra of substrates. Additionally, there is no anaerobic growth by using electron acceptors such as TMAO or thiosulphate, as is observed for *A. fetalis*, which caused the earlier misclassification of *A. fetalis* as a member of *Shewanella*. The spectrum of fatty acids of the Baltic strains is very different from that of *A. fetalis*. Based on the polyphasic approach and in recognition of the very different physiological, chemotaxonomic and biochemical characteristics of the novel isolates, we propose a new genus, *Rheinheimera* gen. nov. Strain OSBAC1^T is proposed as the type strain of the novel species *Rheinheimera baltica* gen. nov., sp. nov., because this strain displayed most of the typical features of the strains isolated. All strains described are considered to belong to the novel species *R. baltica* based on the high DNA–DNA similarity and 16S rDNA sequence comparisons.

Description of *Rheinheimera* gen. nov.

Rheinheimera (Rhein.hei.me′ra. N.L. n. *Rheinheimera* after the German marine microbiologist Gerhard Rheinheimer, in recognition of his work on marine and estuarine bacteria).

Cells are Gram-negative, flagellated, rod-shaped to coccoid, oxidase- and catalase-positive. Growth is aerobic, chemoheterotrophic and occurs at temperatures from 4 to 30 °C, with optimum growth at 20–25 °C. Dominant fatty acids are 16:1ω7*c*, 16:0 and 18:1ω7*c*. Of marine or estuarine origin. NaCl supports growth, but strains are not tolerant of high salinities (> 6%). The genus *Rheinheimera* belongs to the γ-*Proteobacteria*. The type species is *Rheinheimera baltica*.

Description of *Rheinheimera baltica* sp. nov.

Rheinheimera baltica (bal′ti.ca. L. fem. adj. *baltica* from the Baltic Sea, referring to the source of the type strain).

Colonies are circular, smooth, convex, entire and opaque with a blue colour. Colony colour ranges from intense dark blue to light blue. Colonies can be stained entirely or only in the centre of the colony. Cells are variable in shape, ranging from rod-shaped to coccoid, and size (width 0·5–1·5 μm, length 0·9–2·5 μm). They occur typically as single cells with a single polar flagellum. Strains are not halotolerant (no growth at > 6% NaCl); NaCl stimulates growth, but is not required for growth. Optimal growth was observed at 1% NaCl. Growth temperature ranges from 4 to 30 °C, with an optimum at 20–25 °C. Aerobic and chemoheterotrophic. Sugars such as glucose are not metabolized with the production of acid. No growth occurs under anoxic conditions, even in the presence of electron acceptors such as TMAO or thiosulphate. Strains are able to use a wide spectrum of carbohydrates such as D-glucose, *N*-acetyl glucosamine, cellobiose, maltose, starch, glycogen and β-gentiobiose. Strains show good assimilation of glucose, *N*-acetyl glucosamine and maltose. Strains show a broad spectrum of enzymic activities such as β-glucosidase, protease, aminopeptidase, phosphatase (alkaline), leucine arylamidase, trypsin and naphthol phosphohydrolase. Starch, gelatin, Tween 80 and lecithin are hydrolysed. The DNA G+C content is 47·8–48·9 mol%.

The type strain is strain OSBAC1^T (= DSM 14885^T = LMG 21511^T). Strain OSBAC5 has been deposited as DSM 14891.

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