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# Taxonomic and Phylogenetic Studies on a New Taxon of Budding, Hyphal *Proteobacteria*, *Hirschia baltica* gen. nov., sp. nov.

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**Four strains of budding, hyphal bacteria, which had very similar chemotaxonomic properties, were isolated from the Baltic Sea. The results of DNA-DNA hybridization experiments, indicated that three of the new isolates were closely related, while the fourth was only moderately related to the other three. Sequence signature and higher-order structural detail analyses of the 16S rRNA of strain IFAM 1418$^T$ (T = type strain) indicated that this isolate is related to the alpha subclass of the class *Proteobacteria*. Although our isolates resemble members of the genera *Hyphomicrobium* and *Hyphomonas* in morphology, assignment to either of these genera was excluded on the basis of their markedly lower DNA guanine-plus-cytosine contents. We propose that these organisms should be placed in a new genus, *Hirschia baltica* is the type species of this genus, and the type strain of *H. baltica* is strain IFAM 1418 (= DSM 5838).**

Since the first description of a hyphal, budding bacterium, *Hyphomicrobium vulgare* (53), only the following additional genera having this morphological type have been formally described: *Rhodomicrobium* (9), *Hyphomonas* (40), *Pedomicrobium* (1, 14), *Filomicrobium* (45), and *Dichotomicrobium* (23). The taxonomy of these bacteria has been evaluated by using chemotaxonomic characteristics (56), DNA-DNA hybridization (15, 37), genome size determination (28), analysis of nucleotide distribution and DNA base ratio (16), and analysis of RNA cistron similarities (42). Phylogenetically, all of the above-mentioned genera are members of the alpha subclass of the class *Proteobacteria*, and they are not closely related to each other (51).

In this paper we characterize genomically and phenetically four new isolates of budding, hyphal bacteria which have DNA base ratios of 45 to 47 mol%, values which are at least 10 mol% lower than the DNA base ratios of other hyphal, budding members of the *Proteobacteria*.

## MATERIALS AND METHODS

**Enrichment and isolation.** At various times surface water samples (upper 5 cm) of the Kiel Fjord were taken from a boat landing. Different methods were used to enrich for budding, hyphal bacteria immediately after the samples arrived in our laboratory (Table 1). When a microscopic survey indicated the presence of such organisms, streaks were made on solidified M13 medium (44). Colonies were examined microscopically by using the toothpick procedure (23). Pure cultures were obtained within 6 months after sampling.

**Physiological tests.** The isolates were grown in complex M13 medium, which contained 0.025% glucose, 0.025% peptone, and 0.025% yeast extract as carbon and nitrogen sources. Tests for utilization of carbon sources were performed in 100-ml Erlenmeyer flasks containing liquid M9 medium to which a filter-sterilized carbon source was added to a final concentration of 0.1% (wt/vol). Defined M9 medium contained 0.005% $Na_2HPO_4$ and 0.1% $KNO_3$. Methanol and formamide were tested at concentrations of 0.02 and 0.1% (vol/vol). Utilization of nitrogen sources was tested in M9 medium containing glucose as the carbon source. The compositions of the growth media used and the test procedures described above have been described previously for the isolation and characterization of *Pirellula marina* (44). In addition, the tests described below were used. The ability to store poly-β-hydroxybutyric acid (PHB) was tested in M9 medium containing glucose or sodium acetate as the carbon source; glucose and sodium acetate were added to concentrations of 0.1 and 0.5% (wt/vol). To exclude possible repression of PHB storage when there was an excess of phosphorus in the medium, M9 medium was also prepared without $Na_2HPO_4$. Hydrolytic activity was determined on solidified M13 medium to which the appropriate substrate was added to a final concentration of 0.2% (wt/vol). Hydrolysis of casein was indicated by clear zones around the colonies. Hydrolysis of DNA was tested by using the toluidine blue procedure (31). Hydrolysis of lecithin, cellulose, and alginate was tested by using the double-layer technique, with the upper layer containing the substrate (0.2%, wt/vol). Lecithin was added aseptically to autoclaved M13 medium after it was cooled to about 55°C. A positive result was indicated when the released fat caused turbid zones around the colonies. Type MN300 cellulose powder (Macherey-Nagel, Düren, Federal Republic of Germany) was the substrate used for cellulases; cellulase activity was indicated by clear zones around the colonies. Hydrolysis of alginate was demonstrated by flooding petri dishes with a dilute iodine solution (Lugol solution), which resulted in a brown alginate-iodine complex. Clear zones around the colonies indicated that alginate was hydrolyzed.

**Assay for enzymatic activity.** The LRA ZYM Osidase system (API bioMérieux, Montalieu, France) was used according to the instructions of the manufacturer. Bactident aminopeptidase test strips (E. Merck AG, Darmstadt, Federal Republic of Germany) were used to test for aminopeptidase activity. In addition, fluorogenic derivatives of 4-methylumbelliferone (MUF) were used to demonstrate enzymatic activity. The substrates were dissolved in ethylene glycol monomethyl ether (5 mmol/liter), and 1 ml of this solvent was diluted in 50 ml of the appropriate buffer. The following buffers were used: a solution containing 0.2 mol of

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TABLE 1. Dates of sampling and enrichment procedures used for the new strains

| Strain[a] | Date of sampling | Enrichment procedure |
|---|---|---|
| IFAM 1408 | November 1982 | Streaked directly onto M13 medium |
| IFAM 1415 | November 1974 | Glucose (0.05%) added to sample |
| IFAM 1418[T] | October 1982 | *N*-Acetylglucosamine (0.1%) added to sample |
| IFAM 1538 | September 1975 | Petri dish method[b] |

[a] IFAM, Institut für Allgemeine Mikrobiologie, Universität Kiel, Kiel, Federal Republic of Germany.

[b] A layer (about 1 cm) of water agar (1.8% agar in distilled water) on the bottom of a petri dish (diameter, 25 cm) was covered with the sample to a depth of about 2 cm (23).

acetate buffer (pH 5.0) per liter and 5 mmol of $CaCl_2$ per liter for MUF-D-*N*-acetylneuramic acid; a solution containing 0.2 mol of acetate buffer (pH 5.2) per liter for MUF-β-D-galactoside; a solution containing 0.2 mol of $Na_2HPO_4$ per liter and 0.1 mol of citric acid per liter (pH 4.95) for MUF-*N*-acetyl-β-D-glucosaminide; a solution containing 0.2 mol of $Na_2HPO_4$ per liter and 0.1 mol of citric acid per liter (pH 5.2) for MUF-β-glucuronide; and a solution containing 0.1 mol of tris hydrochloride (pH 7.4) per liter for other substrates. Activity was tested by taking a small number of cells from an agar streak with a sterile wooden toothpick and rubbing them onto type 3 MM chromatography paper (Whatman, Maidstone, United Kingdom). A 20-μl portion of the substrate-buffer solution was applied to the bacteria, and after a reaction time of 10 min 20 μl of a saturated $NaHCO_3$ solution was added to enhance the fluorescence (49), which was observed under UV irradiation at 365 nm.

**Resistance to antibiotics.** Resistance to antibiotics was tested by using Bio-Discs (API bioMérieux).

**Analysis of fatty acid composition.** A 50-mg portion of lyophilized bacteria was saponified with 2 N KOH in methanol for 3 h at 70°C. The solution was acidified with 4 N HCl to pH 3 to 4, 150 ml of distilled water was added, and the fatty acids were extracted with 50 ml of light petroleum (boiling point, 40 to 60°C).

Fatty acid methyl esters were prepared by using the method of Minnikin et al. (35, 36), which was modified so that the mixture was incubated overnight at 50°C (M. D. Collins, personal communication). Fatty acid methyl esters were purified by thin-layer chromatography on Silica Gel 60 plates (10 by 10 cm; catalog no. 5735; Merck), which were developed in light petroleum-diethylether (85:15, vol/vol).

**(i) Hydrogenation of unsaturated fatty acid methyl esters.** A sample of fatty acid methyl esters (concentration, about 0.05% [vol/vol] in methanol) was treated with catalyst-activated (5% Pd on charcoal or prereduced $PtO_2$) hydrogen for 20 min at room temperature.

**(ii) Preparation of fatty acid picolinyl esters.** Fatty acid picolinyl esters were prepared by using the method of Harvey (19), as modified by Wait and Hudson (57). The reaction mixture was analyzed by using gas chromatography-mass spectrometry without further purification.

**(iii) Preparation of dimethyl disulfide adducts.** Derivation of fatty acid methyl esters was carried out by using the method of Dunkelblum et al. (10), as modified by Nichols et al. (38).

**(iv) Fatty acid analysis.** Fatty acid methyl esters were analyzed by capillary gas-liquid chromatography with a model 419 Becker gas chromatograph (Packard Instrument Co., Inc., Rockville, Md.) equipped with a flame ionization detector and a model 6300.02 Autolab digital integrator. Samples were chromatographed on a type FFAP-CB capillary column (0.53 mm by 25 m; Machery-Nagel) by using a temperature program of 160 to 210°C (2°C/min). Components were identified by comparison with analytical grade standard methyl esters (Alltech Associates, Inc., Applied Science Div., State College, Pa.). Fatty acid picolonyl esters were analyzed by using gas chromatography-mass spectrometry to locate the branching points of methyl-branched fatty acids, the positions of cyclopropane rings, and the positions of double bonds. Preparations were analyzed by using a Varian model 3700 gas chromatograph equipped with a type OV-1 capillary column (0.32 mm by 50 m) and connected to a Finnigan model MAT 8230 mass spectrometer. The temperature program used was 240 to 280°C (3°C/min), and helium was the carrier gas (6 ml/min). To locate the positions of double bonds in unsaturated fatty acids, dimethyl disulfide derivatives were also analyzed by gas chromatography-mass spectrometry as described above.

**Analysis of hydroxy fatty acids.** Hydroxy fatty acids were prepared by using acid methanolysis with toluene-methanol-concentrated sulfuric acid (5:5:0.2 vol/vol/vol) at 50°C overnight (Collins, personal communication), followed by the treatment described by Minnikin et al. (35, 36). Hydroxy fatty acid methyl esters were analyzed by gas-liquid chromatography (type OV-1; 200 to 270°C; 4°C/min) and were identified by gas-liquid chromatography–mass spectrometry (60).

**Analysis of lipid composition.** For extraction and purification of lipids, lyophilized cell material (50 to 100 mg) was extracted with 30 ml of chloroform-methanol (1:1, vol/vol) for 1 to 2 h in a test tube that was closed with a screw cap. Then 15 ml of chloroform was added, and extraction was continued for 2 h with slight shaking. After filtration through paper filters, crude lipid extracts were fractionated into lipid classes by chromatography on a silica gel column (0.8 by 5.0 cm; approximately 1 g of silica gel; Serva Feinbiochemica, Heidelberg, Federal Republic of Germany). Neutral lipids, glycolipids, and phospholipids were eluted with 10 column volumes each of chloroform, acetone, and methanol. The eluates were taken to dryness in a Rotavapor at 30°C under reduced pressure, dissolved in a small volume of chloroform, and stored in ice until analysis (within 1 h).

**Phospholipid analysis.** Components were separated by two-dimensional thin-layer chromatography on silica gel plates (10 by 10 cm; catalog no. 5554; Merck). Plates were developed by using the method of Collins et al. (4). The following stains were used to detect and identify the components on the plates: iodine vapor for unsaturated lipids and/or lipids containing nitrogen; the Zinzadze reagent (8) for lipids containing phosphate; ninhydrin (0.2% wt/vol) in ethanol for lipids which contained free amino groups or monomethyl-substituted amino groups (lipids with free amino groups appeared as reddish purple spots after 4 to 5 h at room temperature, whereas lipids with monomethyl-substituted amino groups appeared as bluish purple spots after heating at 100°C for 10 min); 1-naphthol reagent (25) for glycolipids; and periodate-Schiff reagent (47) for vincial glycol groups. Phospholipids were also identified by comparison with commercial phospholipid standards (Sigma Chemie).

**Analysis of quinone composition.** Lyophilized cells (50 to 100 mg) were extracted with 45-ml portions of chloroform-methanol (2:1, vol/vol). The resulting extracts were purified by thin-layer chromatography (3). Purified samples were analyzed by reverse-phase partition thin-layer chromatogra-

phy on high-performance thin-layer chromatography plates (type RP18 $F_{254}$; catalog no. 13724; Merck) which were developed with acetone-acetonitrile (80:20 vol/vol) (5). Components were identified by comparing their $R_f$ values with the $R_f$ values of commercial quinone standards (Sigma Chemie), and the identities were confirmed by mass spectrometry (direct inlet system).

**Diaminopimelic acid.** The presence of diaminopimelic acid was tested as described by Rhuland et al. (41).

**Test for nicked 23S rRNA.** rRNAs were isolated from phenol-treated cell extracts and were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis as described by Stackebrandt et al. (52).

**DNA reassociation experiments.** Cells were broken by using glass beads and DNA was purified by using previously described procedures (16). The DNA base ratio was calculated from the transition melting point (33). Lambda DNA was used as a reference. DNA-DNA hybridization was performed on nylon membranes (Hybond-N; Amersham, Braunschweig, Federal Republic of Germany) by using the dot blot procedure (2), which was slightly modified. Portions (0.5 and 1.0 μg) of high-molecular-weight DNA in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were filtered on a nylon membrane by using a filter apparatus (MINIFOLD SRG-96; Schleicher & Schüll, Dassel, Federal Republic of Germany). After air drying, the DNA was baked onto the membrane by exposing it to UV irradiation (254 nm, 5 min). Reference DNA from strain IFAM $1418^T$ (T = type strain) was sheared to an average double-stranded fragment molecular weight of $0.3 \times 10^6$ by two passages through a French pressure cell (Aminco, Urbana, Ill.) at 24,000 lb/in$^2$. DNA was labeled with [$\alpha$-$^{32}$P]dATP by nick translation. Labeling and hybridization were performed by using a Rapid Hybridization Kit (Amersham) according to the instructions of the manufacturer. After stringent washing (7°C below the melting temperature [$T_m$]), the nylon membrane was exposed overnight to X-ray film (Fuji Photo Film Co., Tokyo, Japan). The membrane was then washed at the $T_m$ and again exposed to X-ray film.

**RNA sequence analysis.** Crude rRNA was isolated from strain IFAM $1418^T$, and the sequence of the 16S rRNA was analyzed by using reverse transcriptase (29) and terminal nucleotidyl transferase (6) as described previously (50). The sequence was aligned with reference sequences from a variety of proteobacteria. Binary homology values, which were calculated by using the Microgenie program (Beckman Instruments, Inc., Fullerton, Calif.), were transformed into evolutionary distance values $K_{nuc}$ (24), which were used as the phylogenetic parameter in the Fitch-Margoliash treeing algorithm contained in Phylip version 2 (12).

## RESULTS AND DISCUSSION

**Morphological and physiological properties.** Four strains of yellow-pigmented, hyphal, budding bacteria, which are members of the new genus *Hirschia* described below, were isolated from different samples of surface water taken from Kiel Fjord (Table 1). In morphology (Fig. 1) and life cycle, these four isolates resembled members of the genera *Hyphomicrobium* and *Hyphomonas*. Phenotypic differences between the new isolates and other budding members of the *Proteobacteria* included yellow pigmentation of the colonies, which distinguished the new strains from the white or brownish hyphomicrobia and hyphomonads, from members of the genera *Filomicrobium* (45) and *Dichotomicrobium* (22), which have red colonies, and from members of the

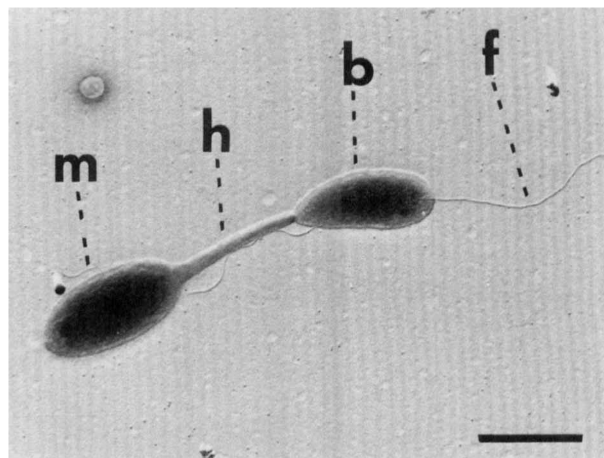


FIG. 1. *Hirschia baltica* IFAM 1415, showing a mother cell (m), a hypha (h), and a bud (b). Note the polar insertion of the flagellum (f). Platinum shadowing. Bar = 1 μm.

genus *Pedomicrobium* (14), which have red to dark brown colonies (Table 2). The colonies of strain IFAM 1408 were mucous, while the colonies of the other strains had a dry, granular appearance.

PHB storage granules, which are present in members of the genera *Hyphomicrobium* (20), *Hyphomonas* (58), *Dichotomicrobium* (22), and *Pedomicrobium* (14), were not observed. All *Hirschia* strains were strictly aerobic and grew well in the presence of artificial seawater (32) at concentrations between 12.5 and 250% (100% artificial seawater corresponds to a salinity of 34.5‰). No growth occurred in the presence of 275% artificial seawater, and only strain IFAM 1538 was able to grow in the presence of 6% artificial seawater. Nitrate was not reduced under anaerobic conditions; glucose was not fermented. The isolates grew on complex media containing peptone, yeast extract, and glucose (M13 medium) and utilized a variety of substrates as carbon sources (Table 3). In contrast to many hyphomicrobia (20, 21), $C_1$ compounds were not metabolized. The patterns of substrate utilization for strains IFAM 1408, IFAM 1415, and IFAM $1418^T$ were very similar (Table 3). Strain IFAM 1538 exhibited a more varied carbon source utilization spectrum in that it utilized glycerol and lactose, and this strain produced α-D-galactosidase, β-D-galactosidase, and β-D-lactosidase (Tables 3 and 4). The *Hirschia* strains hydrolyzed cellobiose by using β-D-glucosidase (Table 4), an enzyme that has not been detected in various strains of *Hyphomicrobium* or *Hyphomonas* spp. Utilization of maltose by strains IFAM $1418^T$ and IFAM 1538 correlated with the presence of α-D-glucosidase, which was not observed in the other isolates. Hydrolytic ability was limited in that only alginate and gelatin were utilized by all of the strains, while casein, cellulose, and lecithin were not hydrolyzed. DNA, starch, and Tween 80 were hydrolyzed by single strains only (Table 3).

**Chemotaxonomic properties.** Our new isolates contained nonhydrogenated ubiquinones with 10 isoprene units (ubiquinone $Q_{10}$). This ubiquinone type nicely separates these strains from hyphomicrobia which contain ubiquinone $Q_9$ (27, 56; Sittig, unpublished data), and from the majority of hyphomonads, which contain ubiquinone $Q_{11}$. An exception is *Hyphomonas jannaschiana*, which, like our isolates, contains ubiquinone $Q_{10}$ (56).

The phospholipid pattern was rather sparse; phosphatidyl-

TABLE 2. Phenotypic characteristics which differentiate the genus *Hirschia* from other genera of budding, hyphal bacteria

| Genus | Morphology | No. and location of hyphae | Flagellation of daughter cells | Color of colonies | Carbon sources utilized | Main quinone component(s) | G+C content (mol%) | PHB storage | Nicked 23S rRNA | Reference(s) |
|---|---|---|---|---|---|---|---|---|---|---|
| *Hirschia* | Rod shaped, elliptical, or ovoid, 0.5–1.0 by 0.5–6.0 µm | 1-2 polar | Monotrichous, polar | Yellow | Sugars, organic acids, amino acids | $Q_{10}$ | 45–47 | − | − | This paper |
| *Hyphomicrobium* | Rod shaped, oval, or egg or bean shaped, 0.3–1.2 by 1.0–3.0 µm | 1–2, polar, subpolar or lateral | 1—3, polar | Brownish | $C_1$ compounds, a few organic acids | $Q_9$[a] | 59–65 | + | − | 20, 51 |
| *Hyphomonas* | Rod shaped to oval, 0.5–1.0 by 1.0–3.0 µm | 1–2, polar | Monotrichous, polar to lateral | Colorless or dull grey | Amino acids | $Q_{10}$, $Q_{11}$[a] | 57–62 | + | + | 51, 56, 58 |
| *Filomicrobium* | Fusiform, 0.5–1.0 by 1.0–4.0 µm | 2 (sometimes 3), polar | Not flagellated | Light red | Organic acids | $Q_9$[a] | 61.9 | + | − | 45, 51 |
| *Dichotomicrobium* | Tetrahedral to spherical, 0.8–1.8 by 0.8–2.0 µm | Up to 4 | Not flagellated | Reddish brown | Organic acids | $Q_{10}$[a] | 62–64 | + | − | 23, 51 |
| *Pedomicrobium* | Oval or spherical, 0.4–2.0 by 0.4–2.5 µm | 1–5 or more, lateral and/or subpolar | Monotrichous, polar to subpolar | Dark brown | Organic acids | $Q_{10}$[a] | 62–67 | + | − | 14, 51 |
| *Rhodomicrobium* | Rod shaped to fusiform, 1.0–1.2 by 2.0–2.8 µm | 1–2, polar, rarely lateral | Peritrichous | Red | Organic acids | $Q_{10}$ | 61.8–63.8 | + | NT[b] | 5, 9, 54, 55 |

[a] Sittig, unpublished data.
[b] NT, Not tested.

TABLE 3. Physiological properties of *Hirschia* strains[a]

| Characteristic | Strain IFAM 1408 | Strain IFAM 1415 | Strain IFAM 1418$^T$ | Strain IFAM 1538 |
|---|---|---|---|---|
| Utilization of the following carbon sources: | | | | |
| Fructose | − | + | − | − |
| Lactose | − | − | − | + |
| Maltose | − | − | + | + |
| Rhamnose | + | − | + | + |
| Glycerol | − | − | − | + |
| Acetate | + | + | + | − |
| Glucuronate | + | − | − | − |
| Lactate | + | + | + | − |
| Propionate | + | + | + | − |
| Pyruvate | + | + | + | − |
| Succinate | + | − | − | − |
| Alanine | + | + | + | − |
| Arginine | − | + | + | + |
| Leucine | − | + | − | + |
| Serine | + | − | − | − |
| Amygdalin | + | − | − | − |
| *N*-Acetylglucosamine | − | − | + | − |
| Hydrolysis of: | | | | |
| DNA | − | + | − | − |
| Starch | − | − | + | + |
| Tween 80 | + | + | + | − |
| $H_2S$ formation from thiosulfate | − | + | + | − |

[a] All of the strains utilized cellobiose, glucose, acetate, butyrate, caproate, lactate, propionate, pyruvate, alanine, asparagine, aspartic acid, glutamic acid, glutamine, isoleucine, proline, and gluconate as carbon sources. The following compounds were not utilized: lactose, xylose, raffinose, ribose, trehalose, adonitol, ethanol, glycerol, mannitol, methanol, methylammonium chloride, formamide, adipate, citrate, formate, fumarate, malate, glycine, histidine, lysine, and valine.

glycerol was the only component. In this respect, our isolates resemble certain *Hyphomonas* strains (Sittig, unpublished data). In contrast, the phospholipid patterns of strains of previously described *Pedomicrobium*, *Filomicrobium*, *Dichotomicrobium*, and *Hyphomicrobium* species are much more complex (Sittig, unpublished data). Glycolipids were absent.

The fatty acid pattern of the *Hirschia* strains (Table 5) is typical of patterns for gram-negative bacteria (26, 30, 48) and hence of little taxonomic significance. The main straight-chain fatty acid component was hexadecanoic acid (22 to 28% of the total fatty acids), while octadecanoic acid (38 to

TABLE 4. Enzymatic activities as determined by using a LRA ZYM Osidase test kit (API ZYM)[a]

| Enzyme | Strain IFAM 1408 | Strain IFAM 1415 | Strain IFAM 1418$^T$ | Strain IFAM 1538 |
|---|---|---|---|---|
| α-D-Galactosidase | − | − | − | + |
| β-D-Galactosidase | − | − | − | + |
| Phospho-β-D-galactosidase | − | − | − | + |
| α-L-Arabinosidase | + | + | + | − |
| β-D-Fucosidase | − | − | − | + |
| β-D-Lactosidase | − | − | − | + |

[a] The following reactions were positive for all strains: α-D-glucosidase, β-D-glucosidase, α-maltosidase, β-maltosidase, and β-D-xylosidase. Negative results were obtained for β-D-galacturonohydrolase, β-D-glucuronidase, *N*-acetyl-α-D-glucosaminidase, *N*-acetyl-β-D-glucosaminidase, α-L-fucosidase, β-L-fucosidase, α-D-mannosidase, β-D-mannosidase, and α-D-xylosidase.

TABLE 5. Fatty acid compositions of *Hirschia* strains

| Fatty acid[a] | % of total fatty acids in: | | | |
|---|---|---|---|---|
| | Strain IFAM 1408 | Strain IFAM 1415 | Strain IFAM 1418$^T$ | Strain IFAM 1538 |
| n14:1d7 | 3.2 | 2.5 | 2.7 | 2.8 |
| n14:0 | 1.1 | 1.6 | 1.6 | 1.4 |
| i15:0 | 0.3 | ND | ND | ND |
| ai15:0 | 0.2 | ND | ND | ND |
| n15:0 | 0.6 | 2.6 | 2.2 | 1.9 |
| n16:1d5 | 4.6 | 2.0 | 1.4 | 4.4 |
| n16:1d9 | 1.6 | 1.2 | 3.3 | 2.4 |
| n16:0 | 22.1 | 22.6 | 24.6 | 28.2 |
| i17:0 | 0.3 | ND | ND | ND |
| n17:1d9 | 0.3 | 0.7 | 0.7 | 0.7 |
| n17:1d11 | ND | 0.5 | 0.6 | 0.9 |
| 9,10cy17:0 | ND | 0.2 | ND | 0.8 |
| n17:0 | 0.8 | 0.8 | 0.9 | 1.8 |
| n18:1d11 | 48.8 | 47.1 | 51.8 | 38.0 |
| n18:2d5,11 | 9.5 | 10.3 | 4.1 | 6.8 |
| n18:0 | 1.9 | 3.2 | 2.1 | 4.7 |
| n19:1d11 | 0.1 | ND | ND | ND |
| 11,12cy19:0 | 1.1 | ND | 0.9 | 0.9 |

[a] n, Normal; i, isobranched; ai, anteiso branched; d, delta (position of double bond from the carboxy group); cy, cyclopropane.
[b] ND, Not detected.

52%) and octadecadienoic acid (4 to 10%) were the main unsaturated fatty acid components. Cyclopropane fatty acids were present in minor amounts (up to 1% of the total fatty acids). Branched-chain fatty acids were absent. Octadecanoic acid was also the main component in three *Hyphomicrobium* strains and two *Pedomicrobium* strains investigated by Eckhardt et al. (11).

The hydroxy fatty acids were very useful for discriminating *Hirschia baltica* IFAM 1418$^T$ from hyphomicrobia and hyphomonads. The main component detected in *Hirschia baltica* IFAM 1418$^T$ was 3-hydroxytetradecanoic acid, which was not detected in either *Hyphomicrobium* strains or *Hyphomonas* strains (Table 6). The hydroxy fatty acids of *Hirschia baltica* IFAM 1418$^T$ were of the unbranched, saturated or unsaturated 3-OH type, as in *Hyphomonas* strains but with longer chains. Hyphomicrobia possessed hydroxy fatty acids of the 2-OH and 3-OH types with relatively long chains.

The cell walls of the new isolates contained *meso*-diaminopimelic acid.

The DNA base compositions (guanine-plus-cytosine [G+C] contents) were 46.1 mol% for strain IFAM 1408, 46.3 mol% for strain IFAM 1415, 45.6 mol% for strain IFAM 1418$^T$, and 47.4 mol% for strain IFAM 1538.

A phenol-treated nucleic acid crude extract from *Hirschia baltica* IFAM 1418$^T$ was separated by sodium dodecyl sulfate slab gel electrophoresis. The bands of large rRNA species (16S and 23S) indicated that an intact 23S rRNA was present. The presence of unnicked 23S rRNAs has also been reported previously for *Hyphomicrobium*, *Filomicrobium*, *Dichotomicrobium*, and *Pedomicrobium* strains, whereas the 23S rRNA of a *Hyphomonas* sp. had a nick which caused cleavage of the molecule into two fragments (16S and 15S) (51). This phenomenon has been reported previously for *Rhodobacter sphaeroides* (34, 61), *Agrobacterium* sp. (7, 18), *Gluconobacter oxydans* (17), *Gluconobacter denitrificans* (17), *Paracoccus denitrificans* (C. Woese, personal communication), *Rhodobacter capsulatus* (Woese, personal communication), *Gemmobacter aquatilis* (43), *Hyphomonas polymorpha* (51), and *Caulobacter crescentus* (52).

**Phylogenetic relationships. (i) DNA hybridization.** The results of DNA pairing experiments indicated that *Hirschia baltica* IFAM 1408, IFAM 1415, and IFAM 1418$^T$ are closely related. As deduced from signals on autoradiograms, the DNAs of strains IFAM 1408 and IFAM 1415 hybridized as strongly with the labeled DNA of strain IFAM 1418$^T$ as the homologous DNA did (Fig. 2). Heteroduplexes formed with strain IFAM 1538 DNA and the labeled probe gave only faint signals, which disappeared after the filter was washed at the ($T_m$) of the DNA. The lower thermal stability of these hybrids indicated that the level of relatedness between strains IFAM 1418$^T$ and IFAM 1538 was significantly lower than the levels of relatedness among the other three strains.

TABLE 6. Hydroxy fatty acid compositions of members of the genera *Hirschia*, *Hyphomicrobium*, and *Hyphomonas*

| Fatty acid[a] | % of total fatty acids in: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Hirschia baltica* IFAM 1418$^T$ | *Hyphomicrobium vulgare* ATCC 27500 | *Hyphomicrobium facilis* ATCC 27484 | *Hyphomicrobium aestuarii* ATCC 27488 | *Hyphomicrobium zavarzinii* ATCC 27495 | *Hyphomonas polymorpha* ATCC 33881 | *Hyphomonas neptunium* ATCC 15444 | *Hyphomonas oceanitis* ATCC 33879 | *Hyphomonas jannaschiana* ATCC 33882 | *Hyphomonas hirschiana* ATCC 33886 |
| 3-OH n11:1 | | | | | | 8.9 | 6.3 | | 13.0 | |
| 3-OH n11:0 | | | | | | 7.7 | 14.0 | | 9.0 | |
| 3-OH n12:1 | 1.5 | | | | | 37.5 | 29.5 | 70.0 | 54.2 | 65.1 |
| 3-OH n12:0 | 15.6 | | | | | 8.1 | 16.3 | 30.0 | 9.1 | 34.9 |
| 3-OH n13:1 | 0.4 | | | | | 19.3 | 7.8 | | 7.9 | |
| 3-OH n13:0 | 1.3 | | | | | 18.6 | 26.3 | | 6.7 | |
| 3-OH n14:1 | 79.3 | | | | | | | | | |
| 3-OH n14:0 | 1.9 | 24.6 | 18.1 | 32.5 | 29.5 | | | | | |
| 3-OH n16:0 | | 8.2 | 37.1 | 14.4 | 18.7 | | | | | |
| 3-OH n17:0 | | 1.1 | 1.2 | 2.7 | 4.3 | | | | | |
| 2-OH n18:1 | | | | 4.7 | 7.8 | | | | | |
| 3-OH n18:1 | | 1.5 | 0.7 | 2.4 | 3.2 | | | | | |
| 3-OH n18:0 | | 39.3 | 6.4 | 21.3 | 29.2 | | | | | |
| 3-OH cy19:0 | | 3.0 | 0.4 | 4.3 | 7.3 | | | | | |
| 2-OH n24:0 | | 7.7 | 15.8 | 6.6 | | | | | | |
| 2-OH n26:0 | | 14.0 | 20.3 | 11.3 | | | | | | |

[a] 3-OH, Fatty acid with an hydroxy group in position 3 (from the carboxy group).

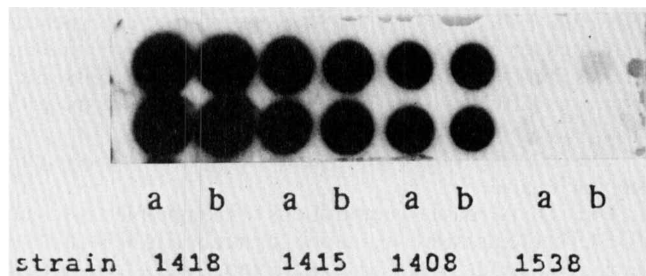


FIG. 2. Autoradiogram of dot blot hybridization with labeled DNA from strain IFAM 1418$^T$. After hybridization the filter was washed at the $T_m$ of the homologous hybrid (72°C). The amounts of target DNA used were 0.5 μg (lanes a) and 1.0 μg (lanes b).

These results were confirmed when labeled DNA from strain IFAM 1538 was used as a probe.

**(ii) 16S rRNA sequence analysis.** Figure 3 shows the almost complete 16S rRNA primary structure of isolate IFAM 1418$^T$; only the 3′-terminal region is missing. The length (1,390 nucleotides) corresponds to 93.4% of the *Agrobacterium tumefaciens* sequence. The sequence was aligned with previously published reference sequences from organisms belonging to the alpha, beta, gamma, and delta subclasses of *Proteobacteria* (see the legend to Fig. 4). A total of 1,125 nucleotides (80.9% of the sequenced rRNA) were used to calculate homology values. The relevant regions are shown in Fig. 3. The parts which were omitted contained highly variable regions and unsequenced stretches of five reference 16S rRNA sequences.

The sequence signature and an analysis of a higher-order structural detail (regions 180 to 220 and 455 to 480) of the 16S rRNA clearly showed that strain IFAM 1418$^T$ is related to the alpha subclass of *Proteobacteria*. Almost all of the signature nucleotides described by Woese (59) for members of this subclass are present. Exceptions included position 233 (U instead of C) and the base pair 502-543 (U · A instead of C · G). The similarity values (Table 7) obtained by using the partial 16S rRNAs from strain IFAM 1418$^T$ and the reference strains clearly indicated that the budding isolate is a member of the alpha subclass of *Proteobacteria*. The binary values were at least 6% higher than the binary values between strain IFAM 1418$^T$ and representatives of the other three subclasses. Figure 4 is an unrooted phylogenetic tree which shows the branching point of strain IFAM 1418$^T$ within the radiation of the alpha subclass of *Proteobacteria*. Strain IFAM 1418$^T$ is a remotely related phylogenetic neighbor of *Hyphomicrobium vulgare* and *Agrobacterium tumefaciens*. However, the limited size of the database did not allow further conclusions about possible membership of the new isolate in the broad phylogenetic group that contains the genus *Hyphomicrobium* and related taxa as defined by 16S rRNA cataloging (51) and a determination of rDNA cistron similarities (42). To investigate the relationships among strain IFAM 1418$^T$, *Hyphomicrobium* spp., *Pedomicrobium* spp., *Filomicrobium* spp., and *Dichotomicrobium thermohalophilum*, the 16S rRNA catalog was extracted from the 16S rRNA sequence of strain IFAM 1418$^T$ (data not shown) and compared with previously published catalogs of the budding organisms mentioned above. The similarity coefficients ranged between 0.46 and 0.53, values which are similar to the values which separate *D. thermohalophilum* from hyphomicrobia (similarity coefficients, 0.42 to 0.55). The low level of relatedness between strain IFAM 1415 and other hyphal bacterial belonging to the alpha subclass has also been determined from the low melting points of DNA-rRNA hybrids (42). Thus, it appears that budding strain IFAM 1418$^T$, like *D. thermohalophilum*, occupies an isolated position within the *Hyphomicrobium* subbranch of the alpha subclass. Furthermore, the position of strain IFAM 1418$^T$ excludes close relationships with other budding members of this subclass (i.e., hyphomonads, *Rhodomicrobium vannielii*, blastobacters, and *Gemmobacter aquatilis*), as well as with other prosthecate organisms (i.e., caulobacters, *Stella* spp. [13], *Ancalomicrobium* spp., *Prosthecomicrobium* spp., and *Prosthecomicrobium*-like bacteria [46]). A close relationship between the genus *Hirschia* and "*Hyphobacter diversus*" can also be excluded. The latter species has been reported to be related to caulobacters and hyphomonads, which, on the basis of 5S rRNA data, are only moderately related to hyphomicrobia (39).

**Conclusions.** Despite morphological similarities to hyphmicrobia and hyphomonads, our four isolates do not fit into any of the previously described genera of budding, hyphal bacteria. The main discriminating phenotypic characteristics of our isolates are the low G+C contents of their DNAs, their hydroxy fatty acids compositions, and their ubiquinone

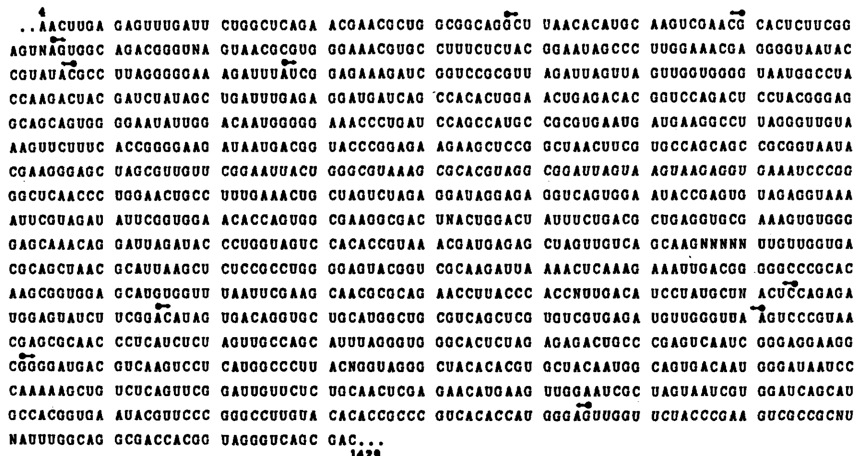

FIG. 3. Partial 16S rRNA sequence for *Hirschia baltica* IFAM 1418$^T$. The numbers refer to the positions in an *Agrobacterium tumefaciens* sequence. The regions between the arrows were used to calculate similarity values. N, Nucleotide of uncertain composition.

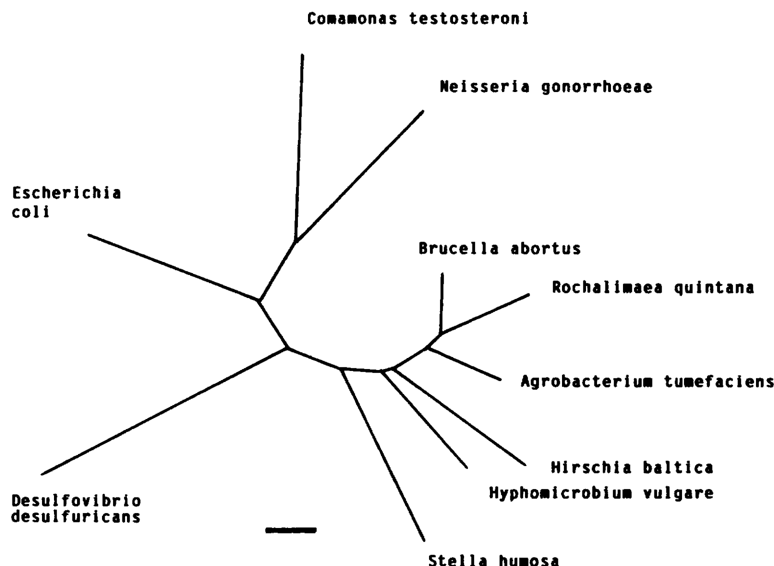


FIG. 4. Phylogenetic tree showing the position of *Hirschia baltica* IFAM 1418$^T$ among members of the alpha subclass of *Proteobacteria*. Bar = 0.02 $K_{nuc}$.

type. Since 16S rRNA sequence analysis data indicated that the phylogenetic relationship between these organisms and the *Hyphomicrobium* subbranch of the alpha subclass of *Proteobacteria* was remote, the creation of the new genus *Hirschia* for the new isolates seems to be justified. Three strains (strains IFAM 1408, IFAM 1415, and IFAM 1418$^T$) are closely related genetically and phenotypically and are described below as *Hirschia baltica*. Strain IFAM 1538 appears to be only moderately related to the other three strains, but its taxonomic status is not settled and should be defined only when additional data are available.

**Description of *Hirschia* gen. nov.** *Hirschia* (Hirsch'i.a. M.L. fem. n. *Hirschia*, honoring Peter Hirsch, a German microbiologist who is an expert on budding and hyphal bacteria). Gram-negative, nonsporeforming, prosthecate bacteria with one or occasionally two polarly inserted hyphae. Reproduction occurs by bud formation at the tips of the hyphae. The buds are polarly monotrichously flagellated. Yellow-pigmented colonies. Aerobic. Chemoheterotrophic. $C_1$ compounds are not utilized as carbon sources. PHB is not stored. The ubiquinone system is a $Q_{10}$ system. Hydroxy fatty acids are of the 3-OH type. 23S rRNA is not nicked. The DNA base ratio of the single species, *Hirschia baltica*, is 45 to 47 mol% G+C. The genus *Hirschia* is a member of the alpha subclass of *Proteobacteria* and is distinctly but remotely related to members of the genera *Hyphomicrobium*, *Filomicrobium*, *Pedomicrobium*, and *Dichotomicrobium*. The type species is *Hirschia baltica*.

**Description of *Hirschia baltica* sp. nov.** *Hirschia baltica* (bal' ti.ca. M.L. fem. adj. *baltica*, pertaining to the Baltic Sea). Cells (without hyphae) are 0.5 to 1.0 by 0.5 to 6.0 μm and are rod shaped, elliptical, or ovoid. The hyphae have a diameter of about 0.2 μm. Colonies are mucous (strain IFAM 1408) or dry. Optimal growth occurs between 22 and 28°C. Artificial seawater is required for growth.

The following carbon sources are utilized for growth: cellobiose, glucose, acetate, butyrate, caproate, lactate, propionate, pyruvate, alanine, asparagine, aspartic acid, glutamic acid, glutamine, isoleucine, proline, and gluconate. No growth occurs with lactose, xylose, raffinose, ribose, trehalose, adonitol, ethanol, glycerol, mannitol, methanol, methylammonium chloride, formamide, adipate, citrate, for-

TABLE 7. Levels of 16S rRNA sequence similarities for *Hirschia baltica* IFAM 1418$^T$ and reference organisms belonging to the alpha, beta, gamma, and delta subclasses of *Proteobacteria*[a]

| Strain | % Homology with: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Hirschia baltica* IFAM 1418$^T$ | *Hyphomicrobium vulgare* | *Agrobacterium tumefaciens* | *Rochalimaea quintana* | *Brucella abortus* | *Stella humosa* | *Neisseria gonorrhoeae* | *Comamonas testosteroni* | *Escherichia coli* |
| *Hyphomicrobium vulgare* MC-750 | 88.4 | | | | | | | | |
| *Agrobacterium tumefaciens* DSM 30105 | 89.0 | 90.4 | | | | | | | |
| *Rochalimaea quintana* Fuller | 88.9 | 87.9 | 92.9 | | | | | | |
| *Brucella abortus* 11/19 | 90.0 | 91.0 | 94.1 | 94.5 | | | | | |
| *Stella humosa* IFAM 1203 | 84.8 | 86.6 | 88.4 | 86.4 | 87.2 | | | | |
| *Neisseria gonorrhoeae* NCTC 8375 | 80.3 | 81.1 | 83.3 | 81.1 | 82.1 | 81.4 | | | |
| *Comamonas testosteroni* ATCC 11996 | 82.1 | 81.4 | 80.8 | 80.2 | 81.4 | 82.8 | 86.8 | | |
| *Escherichia coli* rrnB | 82.5 | 84.4 | 83.8 | 82.3 | 84.0 | 83.3 | 85.1 | 84.1 | |
| *Desulfovibrio desulfuricans* ATCC 27774 | 82.6 | 83.6 | 82.8 | 81.3 | 82.7 | 80.3 | 80.2 | 79.8 | 82.9 |

[a] This analysis was based on a comparison of 1,125 nucleotides.

mate, fumarate, malate, glycine, histidine, lysine, or valine. Alginate, gelatin, and Tween 80 are hydrolyzed, and casein, cellulose, and lecithin are not hydrolyzed. Nitrogen sources are ammonia, glutamic acid, nitrate, and urea. Acetamide, *N*-acetylglucosamine, formamide, and nicotinic acid are not utilized. Ammonia is produced from peptone. Susceptible to ampicillin, oxytetracycline, polymyxin B, streptomycin, and tetracycline. Resistant to bacitracin, nalidixic acid, and oxacillin.

The DNA base composition is 45.6 mol% G+C (type strain) (variable from 45 to 47 mol% G+C, as determined by the $T_m$ method). The cellular fatty acids are composed mainly of straight-chain saturated $C_{16:0}$ acid, straight-chain unsaturated $C_{18:1}$ delta-11 acid, and smaller amounts of straight-chain unsaturated $C_{18:2}$ delta-5,11 acid. Cyclopropane fatty acids occur in minor amounts. Phosphatidylglycerol is the only phospholipid. Glycolipids and branched fatty acids are absent. *meso*-Diaminopimelic acid is the diamino acid of the peptidoglycan.

The habitat is brackish water. The known isolates were obtained from the Baltic Sea (Kiel Fjord). The type strain is strain IFAM 1418 (= DSM 5838).

**Nucleotide sequence accession number.** The 16S rRNA sequence of *Hirschia baltica* IFAM 1418$^T$ has been deposited at the European Molecular Biology Laboratory (EMBL), Heidelberg, Federal Republic of Germany, under the accession number X 52909.

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