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Physiological and Chemotaxonomic Characterization of Filamentous Bacteria Belonging to the Genus *Haliscomenobacter*

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Summary

Nine isolates of filamentous bacteria obtained from activated sludge and morphologically assigned to the genus *Haliscomenobacter* in addition to the type strain of *Haliscomenobacter hydrossis* DSM 1100^T were studied for cellular fatty acid composition, quinone type, physiological characteristics and protein profiles. Two selected representative strains had the MK-7-menaquinone as the major quinone, and the MK-6-menaquinone as minor component. The main components of cellular fatty acids in all strains were the 13-methyltetradecanoic acid (i15 : 0) followed by straight chain unsaturated acids of 16 : 1 and saturated acids of 16 : 0 and 18 : 0. Hydroxylated fatty acids of 3-OH i15 : 0 and 2-OH i15 : 0 were found in higher amount in the type strain of *H. hydrossis*, whereas the isolates from activated sludge produced these acids in minor amounts or traces. Analysis of physiological properties showed phenotypic homogeneity among the tested organisms. From 240 tested organic compounds, only few sugars (D-glucose, D-fructose and a few others) could be utilized as sole carbon source. Standardized protein profiles of the isolates were very similar to the protein profile of *H. hydrossis* DSM 1100^T. The results of the chemotaxonomic characterization are in line with the phylogenetic allocation of the genus *Haliscomenobacter* within the *Saprospira* subgroup of the flavobacter-bacteroides phylum.

Key words: *Haliscomenobacter* – Quinone system – Fatty acid profiles – Physiological characterization – Cellular protein patterns

Introduction

As already pointed out by *Mulder* and *Deinema* (1992) isolates of the genus *Haliscomenobacter* are always present in activated sludge flocs, sometimes in large amounts. Only one species, *Haliscomenobacter hydrossis,* has been described so far (*van Veen* et al., 1973). It is characterized by their straight, thin, needle shaped, sheath forming chains of cells (*Mulder,* 1989; *Mulder* and *Deinema,* 1992) which may protude from the sludge flocs. Despite their frequent detection in activated sludge flocs by microscopic investigations, these filamentous bacteria are difficult to isolate and cultivate, and for this reason, only few studies have been published on the physiology of *H. hydrossis* (*van Veen* et al., 1973; *Krul,* 1977; *Ziegler* and *Dott,* 1990), mainly based on few or even single isolates. On the basis of oligonucleotide cataloging the genus was placed into the cytophaga-flavobacterium group (*Paster* et al., 1985), and in a recent study of small subunit rRNA sequence analysis it was shown that *H. hydrossis* belongs into the Saprospira subgroup of the "flavobacter-bacteroides" phylum which contains *Saprospira grandis, Flexibacter elegans* and *F. sanctii* (*Gherna* and *Woese,* 1992).

Because information about phenetic data of this genus is scarce, and the few results published in literature are mainly based on studies with single isolates and no chemotaxonomic data are available until now, the aim of this study was a comprehensive physiological and chemotaxonomic characterization of a representative set of isolates morphologically assigned to the genus *Haliscomenobacter.*

Materials and Methods

Isolates, growth conditions, morphological characterization and identification

Nine filamentous isolates were obtained from the sewage treatment plants Berlin-Ruhleben and Berlin-Marienfelde (Germany). Methods for isolation and presumptive morphological identifica-

tion are described elsewhere (*Ziegler* et al., 1990). In addition to these isolates (*Haliscomenobacter* sp. VI-39, VI-40, VI-41, VI-42, VI-44, VI-46, VI-48, VI-49, VI-50; see Fig. 1) the type strain of *Haliscomenobacter hydrossis* DSM 1100^T obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, was included. The type of the filamentous bacteria was determined by microscopic examination according to *Eikelboom* (1975) and *Eikelboom* and *van Buijsen* (1981). The morphological and cytological characteristics have been described in detail by *Ziegler* et al. (1990), and the results of nutrient requirements of the isolates have been reported elsewhere (*Kämpfer* et al., 1995). Three of the isolates (VI-40, VI-42, VI-46) have been further used for the development of the *Haliscomenobacter* specific 16S rRNA oligonucleotide probes HHY (*Wagner* et al., 1994).

All strains were grown in liquid GMBN medium. To 960 ml of aqua dest. containing 0.36 g NH_4Cl, 0.15 g $MgSO_4 \times 7\ H_2O$, 0.1 g $CaCl_2 \times 2\ H_2O$, 0.42 g $NaHCO_3$, 0.05 g NaCl, and 0.5 g TRIS, 10 ml of an aqueous solution containing 0.075 g/250 ml $FeCl_3 \times 6\ H_2O$ and 0.075 g/250 ml EDTA were added; pH was adjusted to 7.2, and the mixture was autoclaved at 121 °C. To this solution, 10 ml of a sterile solution containing 0.11 g K_2HPO_4 and 0.08 g KH_2PO_4, 10 ml of a sterile filtered solution containing 0.5 g D-fructose, and 10 ml of a sterile filtered vitamin solution according to *Eikelboom* (1975) was added. All strains were grown for 48 to 72 h at 25 °C at 150 rpm.

Physiological characterization

All strains were tested for 329 physiological features, including 240 carbon source utilization tests, 40 enzymatic tests with chromogenic substrates and 49 sugar acidification tests. The test compounds are listed elsewhere (*Kämpfer* et al., 1991). As a basal medium for all tests, the GMBN medium was used. All test media were prepared double-concentrated (with the exception of NaCl) and 100 µl of each was filled into microtitre plates. The filaments of all strains, grown in 40 ml GMBN, were mechanically disrupted using a Ultraturrax (according to *Kämpfer* et al. 1991), and centrifuged at 15.000 rpm for 10 min. After washing twice with sterile saline and centrifugation steps, 100 µl of the suspensions (MacFarland standard tube No. 5) were filled into each well of the microplates. The microplates were covered with plastic seals, and test results were read photometrically after a seven day incubation at 25 °C. All test results were also read visually.

Quinone and fatty acid analyses

Quinones were studied using the type strain of *H. hydrossis* DSM 1100^T and one isolate (VI-42). Strains were grown aerobically at 25 °C in GMBN medium, and for determination of quinones, thin layer chromatography according to the method of *Collins* (1985) was used. The reference substances for MK-6 and MK-7 were obtained from *Flavobacterium breve* NCTC 11099^T and *Sphingobacterium thalpophilum* NCTC 11429^T (*Dees* et al., 1985).

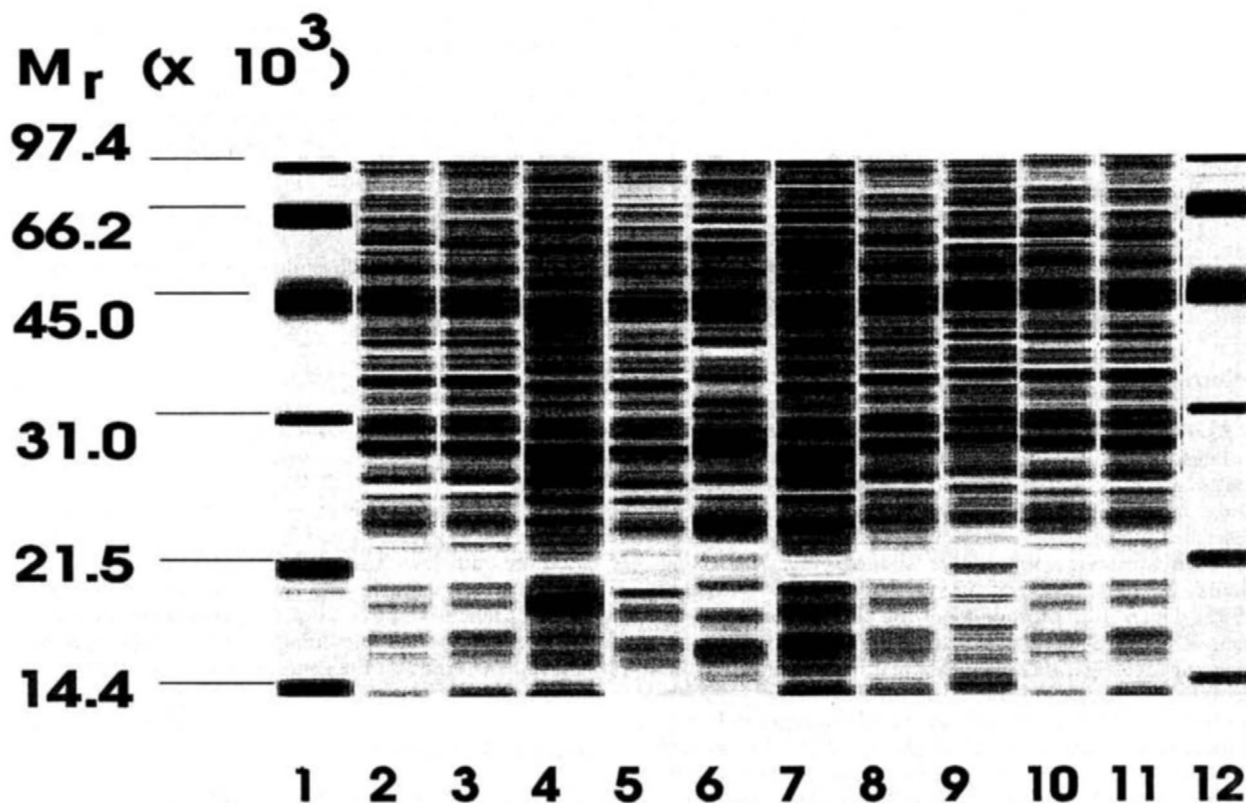

Fig. 1. Normalized cellular protein electrophoretic patterns of *Haliscomenobacter* isolates. Samples were electrophoresed from top to bottom. The lanes contained extracts of *Haliscomenobacter* sp. VI-42, (2), VI-44 (3), VI-48 (4), VI-46 (5), *Haliscomenobacter hydrossis* DSM 1100^T (6), *Haliscomenobacter* sp. VI-41 (7), VI-40 (8), VI-39 (9), VI-49 (10), and VI-50 (11), Lanes 1 and 12 contained reference proteins (molecular masses, M_r, are given).

For extraction and analysis of fatty acids, wet cells harvested from 72-h-cultures in GMBN medium incubated aerobically at 25 °C were studied. Fatty acids were transmethylated with methanolic HCl and fatty acid methyl esters were analyzed by gas liquid chromatography using a Shimadzu GC 9A equipped with a fused silica capillary column (50 m × 0.32 mm) as reported by *Kämpfer* et al. (1992).

Electrophoresis of soluble proteins

Exponential growing cultures in GMBN medium were harvested by centrifugation, washed once with 1 ml of 10 mmol/l Tris-hydrochloride pH 7.3, 5 mmol/l $MgCl_2$, resuspended in 1 ml of the same buffer and the cells were disrupted by sonication (Branson sonifyer, Danbury, C.T., U.S.A.) in an ice-water bath. Lysates were centrifugated in a microfuge at 3000 rev/min for 5 min, and 800 µl of the supernatant fluids were collected. After measuring the protein content (Lowry method), the lysates were distributed in lots containing 50 µg protein and then lyophilized. Dried extracts were resuspended in 25 µl sample buffer (10 ml buffer contained 1.5 ml of 0.5 mol/l Tris, pH 6.8; 4 ml of 10% SDS; 2 ml glycerol; 0.1 ml of 0.4% bromophenol blue; 0.4 ml H_2O; and 1 ml mercaptoethanol), and stored frozen. Five µl of each sample was loaded on the polyacrylamide gels using the Mini-Protean II system (Biorad, München, Germany). Composition, run specifications and staining was done by a modification of the method of *Laemmli* (1970). High range (containing five proteins) and low range standards (containing six different proteins) obtained from BioRad, München, were used as SDS-PAGE standards. Reading of the gels was performed using a computer-controlled video system (Cybertech, Berlin, FRG). Normalization of the electrophoretic traces was performed between the range defined by standard proteins of molecular weights of 116.250 and 14.400 with the GelCompar program (*Vauterin* and *Vauterin* 1992).

Table 1. Cellular fatty acid composition of *Haliscomenobacter hydrossis* DSM 1100^T and nine isolates morphologically assigned to the genus *Haliscomenobacter*

Fatty acid	% of total in: *H. hydrossis* DSM 1100^T	*Haliscomenobacter* sp. (n = 9)
14:0[a]	0.17	0– 0.2[b]
15:0	0.56	0.5– 3.8
16:0	5.68	4.2–11.1
17:0	0.26	0– 0.4
18:0	6.93	3.7– 7.3
19:0	0.11	0
i13:0	0.41	0– 0.3
i15:0	21.06	20.4–41.5
i16:0	2.76	0.6– 2.8
16:1	17.26	7.4–22.5
i17:1	1.77	0– 1.5
a17:1	2.34	0– 1.3
i17:0	1.03	0.2– 1.3
18:1	0.58	0– 0.7
cis-9 18:1	0.23	0– 0.8
cis-9,11 18:1	0.26	0– 1.2
2-OH i15:0	15.49	0– 3.7
3-OH i15:0	22.84	0– 2.3
3-OH i17:0	0.25	0– 0.2

[a] number of carbon atoms: number of double bonds.
[b] range among isolates.

Results and Discussion

Quinones. Both strains studied for quinone profile contained menaquinones of the type MK-7 as major component (70–90%). The menaquinone MK-6 was present, but only in minor amounts (10–30%). The presence of menaquinones is a common trait within genera allocated into the flavobacter-bacteroides phylum (*Collins* and *Jones* 1981). Especially within the genera *Flexibacter, Cytophaga,* and *Flavobacterium,* quinones of the types MK-6 and MK-7 are present (*Collins* and *Jones,* 1981; *Reichenbach,* 1989). In addition, a study on filamentous bacteria from sewage, belonging to the *Flavobacterium-Cytophaga* complex have shown the predominance of the menaquinone system MK-7 and MK-6 in all isolates studied (*Poen* et al., 1984).

Fatty acid analysis. The analysis of all strains revealed characteristic profiles for the *Haliscomenobacter* isolates (Table 1) with four major fatty acids, listed in descending order: 20–41% 13–methyltetradecanoic acid (i15:0), 7–22% cis-9 hexadecenoic acid (16:1), 4–11% hexadecanoic acid (16:0), and 4–7% octadecanoic acid (18:0). Interestingly, the type strain of *H. hydrossis* DSM 1100^T contained high amounts of the hydroxylated fatty acids 3-OH i15:0 and 2-OH i15:0 in addition to minor amounts of 3-OH i17:0. These fatty acids were only present in minor amounts in the isolates (Table 1). It must be stated that the growth of the isolates was very poor in GMBN medium and took several days in order to obtain sufficient biomass for fatty acid extraction. In constrast, cultivation of the type strain resulted in much faster growth and in the production of higher amounts of biomass.

The high content of 13-methyltetradecanoic acid (i15:0) and the presence of 2-OH i15:0 and 3-OH-i15:0 was also found in filamentous isolates of the cytophaga-flavobacterium group studied by *Poen* et al. (1984) and is characteristic for the genera *Flavobacterium* (*Moss* and *Dees,* 1978; *Dees* et al., 1985), *Flexibacter* (*Reichenbach,* 1989), and *Bacteroides* (*Mayberry,* 1980). Similar to the quinone system, the fatty acid profile closely resemble those of other bacteria of the flavobacter-bacteroides phylum sensu *Gherna* and *Woese* (1992).

Physiological tests. All ten strains of *Haliscomenobacter* showed a very similar profile in the physiological characterization. All strains were able to grow with D-fructose, D-glucose, and starch as sole carbon source in GMBN basal medium. The chromogenic substrates pNP-N-acetyl-β-D-glucosaminide, pNP-β-D-glucopyranoside, pNP-phosphate (pH 7.0), pNP-phosphate (pH 8.2), L-alanine-pNA, L-arginine-pNA, glycine-pNA, L-lysine-pNA (pNP = *para*-nitrophenyl-; pNA = *para*-nitroanilide) were hydrolyzed by all strains. N-acetyl-D-glucosamine, D-glucosamine and D-galactose were utilized and pNP-al-

pha-L-arabinopyranoside and pNP-phenyl-phosphonate were hydrolyzed by four isolates. Six isolates were able to use D-xylose as sole carbon source and showed a positive reaction in D-alanine-pNA and L-proline-pNA hydrolysis. Three isolates cleaved the chromogenic substrate pNP-alpha-D-maltoside, and only two were able to grow with L-arabinose. The vast majority of substrates could not be utilized by any of the ten strains: N-acetyl-D-galactosamine, amygdalin, D-arabinose, arabonic acid, p-arbutin, D-cellobiose, dextrane, D-fucose, L-fucose, alpha-D-galacturonate, D-glucarate, gluconate, D-glucosaminic acid, D-glucuronate, glycogen, glycyrrhizinic acid, inulin, lactose, lactulose, D-lyxose, L-lyxose, D-maltose, D-mannose, L-mannose, D-mannoheptulose, alpha-D-melezitose, alpha-D-melibiose, alpha-methyl-D-mannoside, methyl-alpha-D-glucopyranoside, β-methyl-D-xyloside, mucate, 5-oxogluconate, alpha-D-raffinose, L-rhamnose, D-ribose, D-sucrose, salicin, L-sorbose, D-tagatose, D-trehalose, D-turanose, L-xylose, adonitol, D-arabitol, L-arabitol, dulcitol, *meso*-erythritol, glycerol, i-inositol, maltitol, D-mannitol, tributyrine, *meso*-xylitol, acetate, butyrate, caprate, heptanoate, isobutyrate, isovalerate, propionate, sorbinic acid, cis-aconitate, trans-aconitate, adipate, azelate, citraconate, citrate, fumarate, glutarate, glycerate, glycerophosphate, glycolate, glyoxylate, DL-3-hydroxybutyrate, 4-hydroxybutyrate, DL-2-hydroxyisobutyrate, L-2–hydroxyisocaprate, DL-2-hydroxyisovalerate, 2-hydroxyvalerate, DL-isocitrate, itaconate, D-lactate, DL-lactate, L-lactate, levulinate, DL-malate, L-malate, maleate, malonate, mesaconate, mesoxalate, oxaloacetate, 2-oxoglutarate, 2-oxoisocaprate, pimelate, pyruvate, suberate, succinate, D-tartrate, *meso*-tartrate, L-tartrate, tartronate, 1-butanol, 2-butanol, ethanol, 1-hexanol, 1,8-octanediol, acetamidocaprate, acetyl-L-glutamate, acetyl-L-glutamine, acetyl-glycine, acetyl-DL-methionine, acetyl-L-proline, D-alanine, L-alanine, β-alanine, DL-2–aminoadipate, DL-2-aminobutyrate, DL-3-aminobutyrate, DL-2-aminoisobutyrate, 4-aminobutyrate, aminoxyacetate, D-arginine, L-arginine, D-asparagine, L-asparagine, L-aspartate, DL-aspartate, betaine, casein, L-cysteinate, L-citrulline, DL-2,4-diaminobutyrate, diaminopimeltate, 2,3-diaminopropionate, dimethylglycine, D-glutamate, L-glutamate, L-glutamine, L-glycine, glycinanhydride, D-histidine, L-histidine, L-homoserine, DL-delta-hydroxylysine, L-hydroxyproline, DL-isoleucine, L-isoleucine, D-leucine, L-leucine, D-lysine, L-lysine, D-methionine, L-methionine, DL-methioninesulfone, L-norleucine, D-norvaline, L-norvaline, L-ornithine, D-phenylalanine, L-phenylalanine, phenylglycine, D-proline, L-proline, sarcosine, D-serine, L-threonine, D-tryptophan, L-tryptophan, L-valine, butylamine, cadaverine, carnitine, creatine, creatinine, ethalnolamine, ethylamine, urea, hexylamine, putrescine, spermidine, spermine, taurine, trigonelline, tryptamine, tyramine, acetamide, allantoine, cytosine, glycinamide, hippurate, indole-3-acetate, L-leucinamide, oxamid acid, salixylamide, thiamine, uracil, ureidosuccinate, L-valinamide, anthranilate, 3-aminobenzoate, 4-aminobenzoate, benzoate, furan-2-carbonic acid, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-hydroxyphenylacetate, 3-hydrocyphenylacetate, 4-hydroxyphenylacetate, isophthalate, D-mandelate, L-mandelate, phenoxyacetate, phenylacetate, phenyllactate, phenylpropionate, phenylpyruvate, phthalate, protocatechuate, quinate, salicylate, tropate, and vanillate. Growth with D-glucose in the presence of 1%, 2%, 5%, 7%, and 10%, NaCl, and 0.005%, 0.01%, and 0.02% Na-azide was also negative for all strains. None of the tested strains were able to hydrolyze pNP-N-acetyl-β-D-galactosaminide, pNP-D-acetyl-alpha-D-glucosaminide, pNP-β-D-cellobioside, pNP-alpha-L-fucopyranoside, pNP-β-L-fucopyranoside, pNP-β-D-galactopyranoside, pNP-β-D-galacturonide, pNP-alpha-D-glucopyranoside, pNP-β-D-glucuronide, pNP-β-D-lactoside, pNP-alpha-D-mannopyranoside, pNP-β-D-maltoside, pNP-β-D-melibioside, pNP-alpha-L-rhamnopyranoside, pNP-β-D-xyloside, pNP-sulphate, pNP-phosphate (pH 5.4), bis-pNP-phosphate, pNP-phosphoryl-choline, 2-desoxythymidin-5'-pNP-phosphate, acetyl-L-alanine-pNA, acetyl-L-leucine-pNA, benzoyl-DL-arginine-pNA, glutamate-1-pNA, gamma-L-glutamate-pNA, L-glutamate-gamma-3-carboxy-pNA, L-phenylalanine-pNA, L-proline-pNA, succinyl-L-phenylalanine-pNA, and L-valine-pNA, and none of them were able to produce acid from the following carbohydrate: N-acetyl-D-glucosamine, D-arabinose, L-arabinose, arabonic acid, D-cellobiose, dextrane, D-fructose, D-galactose, alpha-D-galacturonate, gluconate, D-glucosamine, D-glucose, D-glucuronate, glycogen, inulin, lactose, lactobionic acid, lactulose, L-lyxose, D-maltose, D-mannose, alpha-D-melezitose, alpha-D-melibiose, alpha-methyl-D-mannoside, methyl-alpha-D-glucopyranoside, β-methyl-D-xyloside, mucate, alpha-D-raffinose, L-rhamnose, D-ribose, sucrose, salicin, L-sorbose, starch, D-tagatose, D-trehalose, D-turanose, D-xylose, L-xylose, dulcitol, m-erythritol, glycerol, i-inositol, maltitol, D-mannitol, D-xylitol, and tributyrine.

Most of the physiological tests gave negative results. Few data on the physiology of *Haliscomenobacter* published until now (*van Veen* et al., 1973; *Krul*, 1977; *Ziegler* and *Dott*, 1990; *Mulder*, 1989; *Mulder* and *Deinema*, 1992). *Krul* (1977) reported, that D-glucose together with some components from trypticase soy broth could be useful as energy source. *Mulder* and *Deinema* (1992) reported that D-glucose can be used as carbon source, whereas glycerol and lactate cannot be utilized, which is in line with our results. In contrast, the utilization of sucrose was reported to be positive, but this could not be confirmed in our study. In addition, *Mulder* (1989) listed glucosamine and lactose, and to a lesser extent mannitol as carbon and energy sources. The utilization of lactose and D-mannitol was not observed for any isolate in this study. As already mentioned above, isolates of the genus *Haliscomenobacter* are difficult to cultivate. It could be shown in a detailed study on the nutritional requirements of these isolates, that D-glucose and D-fructose were good substrates for the cultivation of *Haliscomenobacter* (*Kämpfer* et al., 1995), however a strict dependency was found for the presence of calcium, magnesium, and phosphate in the media for all isolates and high ammonia concentrations (> 2 g l^{-1}) inhibited the growth of *Haliscomenobacter*. Furthermore, *Haliscomenobacter* prefered

a distinct range of phosphorus concentration (0.05–0.2 g l^{-1}), and a low concentration of yeast extract and peptone (*Kämpfer* et al., 1995).

Cellular protein patterns. The normalized profiles of whole cell proteins are shown in Figure 1. All isolates revealed very similar protein profiles with a characteristic lane pattern, indicating a high phenotypic similarity.

Conclusion. This study reports a comprehensive phenotypic characterization of nine Gram-negative filamentous isolates from sewage sludge, morphologically characterized as thin rods in chains enclosed by a small sheath, and assigned to the genus *Haliscomenobacter* in comparison with the reference strain of *H. hydrossis* DSM 1100^T. The homogeneous physiological patterns and cellular protein profiles suggest that the isolates belong to the species *H. hydrossis*. Nevertheless, for the exact species allocation DNA-DNA hybridizations are necessary. The quinone system and fatty acid profiles show the resemblance of these isolates to genera of the "flavobacter-bacteroides" phylum, and therefore support the current phylogenetic classification.

Because isolation and cultivation of these organisms is difficult and time consuming, the use of specific 16S rRNA targeted oligonuleotide probes as reported earlier (*Wagner* et al., 1994) can be very useful for in-situ detection and identification of *Haliscomenobacter*. However, for a more detailed knowledge of the physiology and the ecological role of the genus, more pure culture studies are required.

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