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Diversity and Structure of *Hyphomicrobium* Populations in a Sewage Treatment Plant and Its Adjacent Receiving Lake

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Budding methylotrophic bacteria resembling *Hyphomicrobium* spp. were counted for 12 months in a German sewage treatment plant by most-probable-number (MPN) methods. Influent samples contained up to 2×10^4 cells ml^{-1}, activated sludge consistently contained 1×10^5 to 5×10^5 cells ml^{-1}, and the effluent contained 1×10^3 to 4×10^3 cells ml^{-1}. The receiving lake had only 2 to 12 cells ml^{-1}. Six morphological groups with different growth requirements could be observed among 1,199 pure cultures that had been isolated from MPN dilutions. With dot blot DNA hybridizations, 671 isolates were assigned to 30 hybridization groups (HGs) and 84 could not be classified. Only HG 22 hybridized with a known species, *Hyphomicrobium facilis* IFAM B-522. Fourteen HGs (HGs 8 to 20 and HG 22) were specific for the lake; most others occurred only in the treatment plant. HGs 1, 3, and 26 were found in the activated sludge tank throughout the year, and HGs 27 and 28 were found for most of the year. In summary, it was demonstrated that bacteria with nearly identical and specific morphologies and nutritional types showed a high level of genetic diversity, although they were isolated under the same conditions and from the same treatment plant or its receiving lake. A directional exchange of these genetically different populations was possible but less significant, as was shown by the establishment of distinct populations in specific stations.

Ecological investigations and quantitative determinations of cell numbers of specific bacteria have only been carried out occasionally, i.e., with selective indicator media, or (rarely) where the morphology was so distinct that the genus could be recognized. With certain restrictions, this could be the case with members of the genus *Hyphomicrobium*, gram-negative bacteria which multiply by the production of swarmer cells from the tip of a cellular prostheca, the hypha (10, 19). Even here, other genera with similar morphology exist: *Hyphomonas* (20), *Pedomicrobium* (4), *Hirschia* (26), and *Rhodomicrobium* (13). However, contrary to these morphologically similar genera, hyphomicrobia utilize C_1 compounds, such as methanol, methylamine, formate, etc., for growth (8–10). Presently, eight species have been described (10, 35). With the development of medium 337, a medium containing methanol or methylamine and selective for all hyphomicrobia described so far (16), it became possible to grow, count, and isolate these organisms from natural samples.

Hyphomicrobium spp. have been observed in nearly all habitats that have been investigated (21, 23): soils, groundwater (11), freshwater ponds and lakes, brackish water, marine samples, hypersaline antarctic lakes (12), and sewage treatment plants (17, 27) (Fig. 1). Quantitative studies of the appearance of morphologically recognized members of the genus *Hyphomicrobium* are rare. Throughout 1 year, Staley (30) counted these and other prosthecate bacteria in a polluted stream and found <1 to 15% were hyphomicrobia. In December, he counted as many as 2.4×10^3 ml^{-1}. Stanley et al. (32) studied the numbers of prosthecate bacteria with a *Hyphomicrobium* morphology in fixed samples of pulp mill waste aeration lagoons. They found that hyphomicrobia made up between 0.13 and 2.1% of the total bacterial population.

The physiology of hyphomicrobia has been studied repeatedly (8, 9, 19). It was often noted that hyphomicrobia were capable of denitrification with methanol (1, 28). However, Hirsch (10) stated that denitrification with methanol was only found in some species or strains of the genus *Hyphomicrobium* and that these specific types occurred in sewage or soil. Recently, the presence of denitrifying hyphomicrobia in fluidized bed reactors of drinking water treatment plants (14) and in sewage treatment plants (2, 36) became of special interest because of the necessary removal of nitrate. Coenoses consisting of a *Hyphomicrobium* sp. and a *Paracoccus* sp. strain were quite efficient in the removal of both the methanol and the nitrate (2, 36). A fundamental study of the *Hyphomicrobium* denitrifying capacity showed that these bacteria grew well anaerobically and with identical rates in the presence of methanol and either NO_3^- or NO_2^- (33). Recently, a coimmobilized mixture of a *Hyphomicrobium* sp. and a *Methanosarcina*-like bacterium was very efficient in simultaneous denitrification and methanogenesis (15).

The present work was initiated (i) to take advantage of highly selective enrichment and isolation methods combined with the characteristic morphology for the determination of *Hyphomicrobium* cell numbers in a sewage treatment plant and (ii) to study the structure, diversity, and possible sequence of *Hyphomicrobium* populations in the incoming raw sewage, in the activated sludge tank, in the effluent, and in the receiving freshwater lake. Cell number determinations were based on the most-probable-number (MPN) method. For the study of species diversity, 1,199 strains were isolated and further divided into defined groups by morphological and especially molecular genetic methods. For reference purposes, we also studied some known *Hyphomicrobium* species and a few other isolates from the culture collection.

Sampling of the four sites occurred monthly over a 1-year period to detect possible seasonal changes. Since the four sites were unidirectionally interconnected, it also became possible to study the influences of each preceding site on the next one. If there were distinct hyphomicrobia in each of these sites, they

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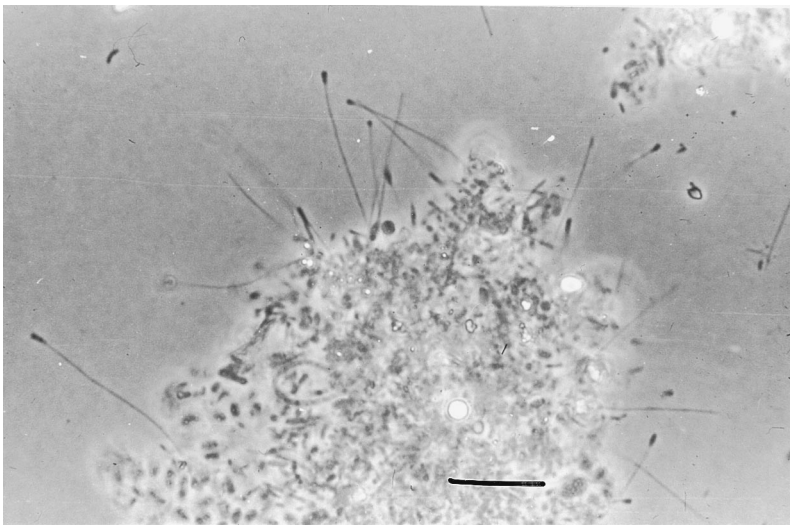

FIG. 1. Activated sludge floc with several *Hyphomicrobium* cells attached to it. Bar, 10 μm.

could conceivably be used as indicator organisms for specific conditions within these sites. Thus, the fundamental importance of the present study is that the passage of morphologically distinct bacteria through a sewage treatment plant could be monitored qualitatively and quantitatively. Especially significant was the detection of a possible genetic heterogeneity among the morphologically and nutritionally similar bacteria present in different locations.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. The bacterial strains used as a reference for DNA-DNA hybridizations are listed in Table 1. They were cultivated at 30°C in medium 337-B4, which consisted of mineral salts medium 337-B (16) with methylamine (3.38 g liter^{-1} [wt/vol]) but without sodium gluconate and L-lysine. For MPN enrichment cultures, medium 337-B4 was amended with cycloheximide (200 mg liter^{-1}) to reduce growth of protozoa and algae. Pure cultures of *Hyphomicrobium* spp. isolated from the MPN tubes were further cultivated either in medium 337-B4 or in medium 337-B5. The latter medium corresponded to medium 337-B4 but had the following additions: Bacto Peptone (0.25 g liter^{-1}), Hutner's mineral salts (20 ml liter^{-1} [3]), and vitamin solution no. 6 (10 ml liter^{-1} [29]).

TABLE 1. Bacterial strains used in this study

Strain[a]	Reference
Hyphomicrobium vulgare IFAM MC-750^T (= ATCC 27500^T)	10
Hyphomicrobium aestuarii IFAM NQ-521gr^T (= ATCC 27483^T)	10
Hyphomicrobium hollandicum IFAM KB-677^T (= ATCC 27498^T)	10
Hyphomicrobium facilis IFAM B-522 (= ATCC 27484)	10
Hyphomicrobium facilis subsp. *tolerans* IFAM CO-558 (= ATCC 27491)	10
Hyphomicrobium zavarzinii IFAM ZV-620	10
Hyphomicrobium denitrificans IFAM HA-905 (= TK 0415^T = DSM 1869 = *Hyphomicrobium* X)	35
Hyphomicrobium "*variabile*" IFAM 1761	34
Hyphomicrobium sp. IFAM strains	
SW-808	7
T-854	7
WI-926	7
1168	7
1465	7
GF-849	7

[a] IFAM, Institut für Allgemeine Mikrobiologie, University of Kiel, Kiel, Germany; ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Sewage treatment plant and sampling procedures. The treatment plant in Plön (Schleswig-Holstein, Germany) received 2,500 to 3,500 m^3 of mainly domestic wastewater per 24 h in the summer, but only 2,500 m^3/24 h in winter. The biological oxygen demand of incoming wastewater ranged from 150 to 350 mg liter^{-1}, the suspended solid concentrations ranged from 100 to 250 mg liter^{-1}, and the chemical oxygen demand ranged from 400 to 800 mg liter^{-1}. The wastewater treatment processes involved four steps: (i) mechanical screening, (ii) transfer to the primary settling tank with an overall volume of 850 m^3, (iii) treatment in the activated sludge tank (volume, 2,160 m^3) with simultaneous chemical phosphate elimination (with $FeCl_3$), and (iv) transfer to the secondary settling tank with a returned sludge rate of approximately 1.0. The aeration time in the activated sludge tank was 2 h (nitrification period), followed by 2 h without aeration (denitrification period). The sludge age was 14 to 20 days. Thus, the biological treatment process can be characterized as a combination of simultaneous and intermittent nitrification and denitrification processes. The effluent receiving water body was a lake, Kleiner Plöner See. The waste-activated sludge (ca. 1,000 kg of dry matter day^{-1}) was further treated in an anaerobic digester together with the primary sludge, followed by mechanical dehydration with a filter belt. The turbid water separated in the waste sludge treatment process (i.e., water from the anaerobic digester and filtrate from the mechanical dehydration process) was pumped back into the primary settling tank. Therefore, possibly recycled bacteria from the waste sludge treatment could be detected in the effluent of the primary settling tank. During the sampling period, the performance of the process described above was without problems. The sludge index was almost always below 100 ml g^{-1}, with no bulking or other activated sludge problems. The suspended solids concentration in the activated sludge tank was on average 6 g liter^{-1}; in the effluent of the secondary settling tank, it amounted to 20 mg liter^{-1}. The nitrification-denitrification process worked well during the whole sampling period.

In a monthly screening program over the course of a year (1984 to 1985), water samples were taken from three locations of the treatment plant: (i) the influent into the activated sludge tank (effluent of the primary settling tank), (ii) the activated sludge tank, and (iii) the effluent of the secondary settling tank, which was directly connected with the lake. Samples from the receiving lake were taken approximately 1 km from the plant. In all cases, 500-ml samples were collected between 8:00 and 9:00 a.m. at an average depth of 20 cm with sterile 1-liter bottles. They were chilled over ice until studied in the laboratory approximately 1.5 h later.

MPN determinations and enrichment cultures. To estimate the total size of the viable *Hyphomicrobium* community, medium 337-B4 with cycloheximide (200 mg liter^{-1}) and an MPN procedure were employed. Chilled samples (100 ml) were blended at low speed for 4 min (Waring Blender, Eydam, Kiel, Germany). Depending on the *Hyphomicrobium* concentration in these habitats as determined in preliminary tests, dilutions were made in 1:1 steps in the case of the activated sludge (16 to 22 replicates) and the lakewater samples (10 to 16 replicates). Otherwise, dilutions were 1:10-fold (10 replicates). The large number of replicates and low dilution rates in the case of the activated sludge and the lakewater were used in order to minimize the standard deviation. MPN tubes

were incubated in the dark for 6 to 12 weeks at 20 ± 2°C. The appearance of hyphomicrobia (indicated by the presence of hyphae and buds) was determined by microscopy, and their numbers were then calculated according to the MPN method as previously described (22).

Isolation of pure cultures. Either the three highest dilutions (for activated sludge and lakewater) or the highest dilution (influent and effluent) which contained hyphomicrobia only was streaked on two plates with medium 337-B4 and incubated for 2 to 12 weeks at 20°C in the dark. Colonies containing a *Hyphomicrobium* cell morphology were purified on medium 337-B4 and further maintained or grown for experimental purposes on medium 337-B4 or medium 337-B5.

Preliminary characterization of the isolates. All pure cultures grew with methylamine as a carbon and nitrogen source. As a prerequisite for the hybridization procedure, the isolates were divided into six preliminary groups (PGs) on the basis of morphological and growth characteristics such as cell size and shape, cell length, number and branching of hyphae, colony color and texture, growth within 2 weeks or only after 2 months, and growth requirements for components of medium PYGV (29). The most important properties of these preliminary groups are as follows.

In PG I, mother cells developed up to five hyphae from one cell pole. The hyphae were often branched up to four times. In older liquid cultures, mother cells often shed their hyphae. Growth of all of these strains was stimulated with vitamin solution no. 6 and/or Hutner's basal salts. Peptone also stimulated growth, but the presence of methylamine was mandatory. All 264 strains belonging to this group came from Kleiner Plöner See.

In PG II, the cells resembled those of *Hyphomicrobium vulgare* IFAM MC-750^T. Colonies were very rigid, but smooth variants occurred occasionally. None of the PYGV components stimulated the growth of any of these strains. All 228 isolates of this PG came from the sewage treatment plant.

For PG III, the overall description corresponded to that of PG II organisms, with the exception that all colonies had a slimy texture. The 199 isolates of PG III came from all four sampling sites.

In PG IV, the members of the group resembled those of PG II, with the exception of having a more variable cell shape (pear, banana, or drop shapes). The colonies were characteristically conical and had concentric rings. The 43 strains of this group were isolated from the sewage treatment plant.

In PG V, the cells were often pleomorphic and aggregated strongly to large packages but otherwise resembled those of PG IV. However, the colonies were never round but always polygonal, gray, and extremely rigid. With one exception, all 29 strains came from the sewage treatment plant.

In PG VI, the group differed from the other five groups with respect to nearly all of the aforementioned properties. The cell and colony morphologies were similar to those of *H. vulgare* IFAM MC-750^T, but mother cells were smaller (0.5 to 1.0 μm), and the usually unbranched hyphae were shorter (3 to 4 μm). Motile daughter cells were never found. Growth in liquid media was very slow and occurred only in medium 337-B5, with cell numbers not exceeding 10^8 ml^{-1}. With the exception of nine strains, all 436 isolates of this group were obtained from the sewage treatment plant.

DNA isolation. *Hyphomicrobium* cells were collected from two to five agar plates with a sterile spatula, transferred to a cap with 1-mm-diameter glass beads (Sigma, Deisenhofen, Germany), and homogenized and washed with 1.0 ml of saline EDTA (150 mM NaCl, 100 mM EDTA [pH 8]). The cells were again resuspended in a solution containing 380 μl of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.5]), 8 μl of 25% (wt/vol) sodium dodecyl sulfate (SDS; to give a final concentration of 0.5% [wt/vol]), and 12 μl of proteinase K (0.5% [wt/vol]). After homogenization (every 10 min) and incubation for 1 h at 60°C, SDS (24 μl [25%]) was added to give a final concentration of 2% (wt/vol). After incubation for 1 h at 60°C, 5 M NaCl was added to obtain a final concentration of 1 M. Cells were lysed furthermore by addition of 500 μl of phenol (saturated with 0.1× SSC) and kept for 30 min at 60°C. The cell debris was then pelleted for 15 min at 15,000 × *g* (4°C).

The isolation procedure of Sambrook et al. (25) for high-molecular-weight genomic DNA was followed, except for two ethanol precipitation and washing steps. The DNA was redissolved in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and its concentration and purity were determined spectrophotometrically.

DNA manipulations and hybridizations. Procedures for agarose gel electrophoresis, dot blotting (Hybond N filter; Amersham, Little Chalfont, Buckinghamshire, United Kingdom), and hybridization of radiolabelled DNA were done as described previously (25). Labelling of DNA ([α-^{32}P]dATP) was performed with a nick translation reagent kit from Gibco BRL (Eggenstein, Germany) according to the manufacturer's protocol. Labelled DNA was purified with a Geneclean kit (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions and then was redissolved in 30 μl of double-distilled water. The filters were loaded with 1 μg of DNA per spot. The presence of DNA on the filters was demonstrated with a universal eubacterial rRNA gene probe (positions 787 to 803). According to the published GC contents of known hyphomicrobia, the prehybridization and hybridization conditions were as follows: prehybridization (66°C for 2 h) and hybridization (66°C for 12 to 24 h) were performed in 6× SSC–5× Denhardt's solution (25)–0.5% SDS. Filters were subsequently washed at 66°C in 2× SSC (15 min), 2× SSC with 0.1% SDS (30 min), and twice in 0.1× SSC to remove the unbound DNA.

RESULTS

MPN determinations and isolation of hyphomicrobia. It is important to mention again that the sewage treatment plant functioned normally during the investigation time and that the composition of incoming raw municipal sewage remained reasonably constant—two prerequisites for the ecological study reported here. Conditions in the activated sludge tank were quite constant, with pH varying between 6.8 and 7.3 and a temperature of 9 to 18°C (monthly data not shown). The conditions in the receiving lake were much less stable, with pH between 7.5 and 9.0 and the temperature varying from 0 to 20°C.

The first enrichments from the activated sludge tank did not contain cycloheximide, and the dilutions were only carried out until 1.25×10^{-6}. When microscopy revealed large numbers of flagellates in these enrichments, cycloheximide was added to further enrichments at a concentration which was not inhibitory to other known hyphomicrobia (200 μg ml^{-1}). Also, dilutions were carried further because of too-high numbers of bacteria in the activated sludge. Only a few hyphomicrobia could be detected in such enrichments within the first 2 weeks. Later, their numbers increased and then remained constant after 2 months. Most of the enrichments with lake water did not yield hyphomicrobia before 2 months of incubation time and did not show visible increases after 3 months. The evaluations of the MPN experiments were therefore done after 3 months of incubation. The results are shown in Table 2. Total numbers of hyphomicrobia in the influent varied considerably during the sampling period, while those in the activated sludge tank remained remarkably constant at about 10^5 ml^{-1} throughout the year. MPNs of hyphomicrobia in the lake were extremely low compared with those for the treatment plant. The three lowest-dilution tubes with hyphomicrobia were streaked onto 337-B4 agar plates, and colonies with hyphomicrobia were further purified.

Genetic diversity of the isolates as revealed by hybridization experiments. The hybridization experiments were done with 755 strains of PG I to PG V; strains of PG VI did not render sufficient DNA because of scant and slow growth. Likewise, eight strains of PG I to PG V did not yield DNA at all, and thus, these too could not be included in the hybridizations. Because of the large number of strains to be tested, the hybridizations were performed in four stages, in which the result of each preceding stage determined the strain selection for the next one. The following strategy was applied. Stage 1 involved all strains of PG I and additionally the reference strains listed in Table 1. In this way, the DNAs of 278 strains were placed on six filters. Fifteen hybridization experiments were performed with radioactively labelled DNA of the reference strain, *Hyphomicrobium facilis* IFAM B-522, and labelled DNA of 14 strains of PG I. For the first 10 hybridization experiments, those strains were selected which were not placed into any previous hybridization group (HG), followed by four experiments with strains that had already been classified. Stage 2 involved all strains of PG II, as well as the reference strains of Table 1, the 10 new reference strains which determined the 10 HGs of PG I, and all isolates of PG I that could not be placed into any of the first 10 HGs. Thus, 12 hybridization experiments were performed with members of PG II and the reference strains of Table 1 according to the strategy of stage 1. These experiments resulted in eight new HGs. Stages 3 and 4 were performed according to the protocol described above, with all strains of PG III or PG IV plus PG V, respectively.

Altogether, 671 strains could be placed into 30 HGs (Table 3). The remainder of 84 strains from PG I to PG V did not

TABLE 2. Total numbers of *Hyphomicrobium* organisms from the four sites as determined in sample dilutions and with the MPN method

Date (mo/day/yr)	No. of cells ml^{-1} in samples from[a]:			
	Influent	Activated sludge tank	Effluent	Kleiner Plöner See
12/15/84	ND[b]	$>3 \times 10^5$[c]	ND	8
1/15/85	ND	$>3 \times 10^5$	ND	6
2/20/85	ND	$>3 \times 10^5$	ND	ND
3/21/85	ND	4×10^5	ND	6
4/18/85	ND	3×10^5	3×10^3	9
5/21/85	2×10^4	3×10^5	2×10^3	3
6/19/85	ND	1×10^5	3×10^3	2
7/24/85	4×10^1	2×10^5	1×10^3	5
8/21/85	1×10^3	3×10^5	3×10^3	11
9/19/85	1×10^3	4×10^5	4×10^3	12
10/28/85	1×10^4	5×10^5	1×10^3	2
11/25/85	ND	5×10^5	ND	7

[a] The factors of the 95% confidence range were 2.57 for the influent and effluent, 1.55 for the lake, and <1.55 for the activated sludge tank.
[b] ND, not determined.
[c] Since the maximum sample dilutions of 1.25×10^{-6} were still overgrown with hyphomicrobia, the actual numbers exceeded the minimum of 3×10^5 cells ml^{-1}; later samples were diluted further.

hybridize in this procedure. Of all of the HGs tested, only HG 22 hybridized with a known *Hyphomicrobium* species, *H. facilis* IFAM B-522.

Spatial distribution and seasonal diversity of HGs. The presence and location of members of the HGs are shown in Table 3. HGs 1 to 7 (from PG II) came exclusively from the treatment plant. HGs 1 and 3 (172 strains) made up the major part of PG II. They were found in the activated sludge tank throughout the year. PG VI accounted for 55% of all hyphomicrobial isolates from the activated sludge; HGs 1 and 3 accounted for 20%. HGs 8 to 17 (PG I) as well as HGs 18 to 20 and 22 (PG III) were derived exclusively from the lake. Among these, HGs 8 and 9, with a total of 165 strains, made up 49% of all lake strains and could be isolated throughout the year.

Of special interest was HG 25, with 20 strains that hybridized with IFAM 1465, a strain isolated previously from another sewage treatment plant. The hyphomicrobia of HG 25 that were studied by electron microscopy were found to have helically twisted hyphae (7), as was the case with IFAM 1465 (Fig. 2). The members of HG 25 were found at all four sampling sites. Their highest incidence was in the influent. The same holds true for the 123 isolates from HG 26 which came from PG II and PG III. These were dominant (40%) in the influent (Table 4).

A total of 37 strains of HG 27 (from PG IV) were found only in the sewage treatment plant but never exceeded 9% of all

TABLE 3. HGs of *Hyphomicrobium* spp. in four habitats

HG	No. of isolates	PG[a]	Mo of occurrence in[b]:			
			Influent	Activated sludge tank	Effluent	Kleiner Plöner See
1	140	II	8, 10	All year	4–6, 8, 10	—
2	10	II	—	7, 8	4, 6, 10	—
3	32	II	7, 9, 10	All year	5, 8	—
4	8	II	—	2, 4, 8, 9, 11	4	—
5	2	II	—	4, 8	—	—
6	3	II	—	7, 11	—	—
7	2	II	5	9	—	—
8	105	I	—	—	—	All year
9	60	I	—	—	—	All year
10	14	I	—	—	—	9
11	14	I	—	—	—	3, 5, 6, 8, 10, 11
12	18	I	—	—	—	12, 3, 4, 6, 7, 9, 10
13	1	I	—	—	—	6
14	3	I	—	—	—	3, 4
15	3	I	—	—	—	3, 7, 10
16	2	I	—	—	—	1, 4
17	4	I	—	—	—	5, 7, 8, 11
18	17	III	—	—	—	12, 3–5, 7, 8, 11
19	6	III	—	—	—	6–9
20	1	III	—	—	—	9
21	8	III	—	4, 6, 11	5	7
22	4	III	—	—	—	4, 8, 11
23	1	III	—	11	—	—
24	2	III	—	7, 9	—	—
25	20	III	7–9	8	5–7, 10	4, 8, 11
26	123	II and III	4, 6–10	All year	4, 6–10	12, 4, 7–11
27	37	IV	5	2, 3, 6, 7–11	7, 8, 10	—
28	28	V	5	4–8, 10–11	7, 9, 10	7
29	2	IV	—	—	7	—
30	1	V	—	—	9	—
No HG			11 isolates	24 isolates	5 isolates	52 isolates

[a] PGs were added for comparison.
[b] Lines and boxes mark HGs with distinct distributions. —, not present.

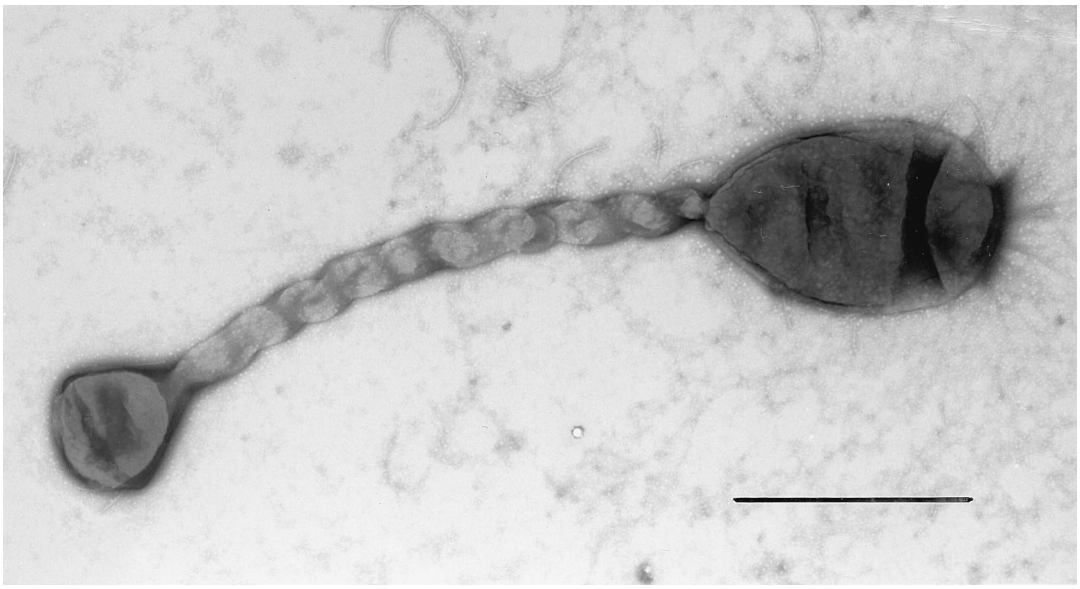

FIG. 2. *Hyphomicrobium* sp. strain IFAM 1465, from a sewage treatment plant, showing helically twisted hypha. Bar, 1 μm.

hyphomicrobia. Of these, 22 isolates had their seasonal maximum between July and October. A very similar spatial distribution and seasonal diversity were observed with HG 28 (from PG V).

DISCUSSION

Counting of hyphomicrobia in the samples by direct microscopy was not possible because of the presence of particulate matter (in treatment plant samples) or because of too-low numbers (in lakewater samples). Therefore, the MPN method was employed by which numbers were calculated in terms of the presence of hyphomicrobia in dilution enrichments (Table 2). This was not done without reservations, for the following reasons. (i) Activated sludge homogenization of the samples for 4 min at room temperature reduced the size of aggregates to around 10 microbial cells per floc, which means that the numbers given in Table 2 could be somewhat low. Further homogenization resulted in lower cell numbers. (ii) Dilution of *Hyphomicrobium*-containing samples may pose special problems because of the tendency of some hyphomicrobia to stick to glass walls. (iii) Activated sludge normally contains more than 10^{10} cells ml^{-1}. Consequently, the last dilution with hyphomicrobia (5.3×10^6 ml^{-1}) should have had at least approximately 2×10^3 other microorganisms ml^{-1}. It can be assumed that the presence of these stimulated the growth of some hyphomicrobia, as was shown by the PG VI organisms. Further dilution would have separated these from the hyphomicrobia and thus prevented hyphomicrobial growth at the lower dilutions. For these reasons, the actual *Hyphomicrobium* numbers could have been higher. However, the distribution of positive enrichment tubes, especially the frequency of negative tubes within the dilution series, agreed to a high degree with the statistical distribution that could be predicted if hyphomicrobia could be positively enriched at any dilution rate.

TABLE 4. Percentage of total *Hyphomicrobium* isolates in each HG over the whole year

HG	% of total *Hyphomicrobium* isolates in samples from[a]:				PG
	Influent	Activated sludge tank	Effluent	Kleiner Plöner See	
1	9	17	12	—[b]	II
2	—	<1	12	—	II
3	7	3	6	—	II
8	—	—	—	31	I
9	—	—	—	18	I
10	—	—	—	4	I
11	—	—	—	4	I
12	—	—	—	5	I
18	—	—	—	5	III
25	9	<1	7	3	III
26	40	11	12	5	II and III
27	2	4	9	—	IV
28	2	3	4	<1	V
?[c]	24	3	7	15	—

[a] The total numbers of isolates from the four sites were as follows: influent, 45; activated sludge tank, 745; effluent, 67; and Kleiner Plöner See, 342. The numbers of strains tested from the four sites were as follows: influent, 43; activated sludge tank, 334; effluent, 53; and Kleiner Plöner See, 333.

[b] —, not present.

[c] ?, strains which could not be classified with any of the HGs.

The MPN dilution tubes were used as enrichments. In the case of the treatment plant samples, up to three morphologically different hyphomicrobia could be isolated from each tube. These usually were also classified in different hybridization groups. In the lake water samples, there was significantly less morphological diversity: hyphomicrobia of PG I were usually detected in sufficient numbers only after 3 months, while most of PG III had already appeared within 2 months. The late appearance of slowly growing PG I hyphomicrobia could mean that they were to some degree dependent on interactions with components of the microbial population which had been diluted out. Conceivably, high-dilution enrichments of other bacteria and longer incubation times might generally result in the isolation of many new taxa in which only one species is known so far (e.g., as in the cases of *Ancalomicrobium* [29] and *Prosthecobacter* [31] species). The results shown in Tables 2, 3, and 4 allow the interpretation of a population dynamic in which the hyphomicrobia of the activated sludge consisted partly of an

established and growing resident population and partly of organisms arriving from the influent.

Members of PG VI, although budding, did not have motile cells, which does not agree with the *Hyphomicrobium* type species description. This may be an indication of the presence of a new genus within PG VI. The absence of daughter cell motility may have been a response to specific survival conditions in the activated sludge tank. Only those cells which settle together with flocs in the settling tank (and thus could be returned to the activated sludge) can establish and maintain an independent, stable population in the activated sludge. The hydraulic retention time there is more than 10 times lower than the generation time of the activated sludge bacteria.

The dot blot hybridizations of this study were carried out under stringent conditions (i.e., at 66°C, a temperature which was 10 to 16°C below the average midpoint temperature of known *Hyphomicrobium* strains [6]). In the cases of HGs 1 and 8, stringency was tested by subsequent washings in three to four steps at temperatures up to 80°C. No further differentiation could be achieved with this procedure. Our dot blot hybridization data also supported previous results (5, 24). It was most remarkable that strains *H. vulgare* IFAM MC-750^T, *Hyphomicrobium aestuarii* IFAM NQ-521gr^T, *Hyphomicrobium hollandicum* IFAM KB-677^T, *Hyphomicrobium zavarzinii* IFAM ZV-620, *Hyphomicrobium denitrificans* IFAM HA-905 (= TK 0415^T = *Hyphomicrobium* X), SW-808, T-854, and IFAM 1761 hybridized only with themselves but not with the 755 isolates investigated in this study. By hybridization, a relationship of the soil isolate *H. facilis* IFAM B-522 with members of HG 22 (found only in lake water samples) and with IFAM strains CO-558, GF-849, and 1168 could be detected. Members of HG 22 could have been soil strains that were washed into the lake. Also, hybridizations revealed the identity of a forest pond water isolate, IFAM WI-926, with HG 24 (found only in the activated sludge tank) and the identity of members of HG 25 with IFAM 1465. Cells of those strains of HG 25 (from activated sludge) which were subjected to electron microscopy had helically twisted hyphae (Fig. 2), which was a stable characteristic which could not be changed by culture conditions. So far, too few phenotypic and genotypic properties of the strains with helically twisted hyphae are known to warrant a description of a new taxon, although Gliesche et al. (7) observed that hyphomicrobia with twisted hyphae had four bacteriophages which did not attack any of the other 40 *Hyphomicrobium* strains tested. Cells with twisted hyphae have been described previously (7, 18), and in some cases they came from sewage treatment plants; in other cases, they came from soil. Also surprising was the fact that *H. hollandicum* IFAM KB-677^T from a sewage treatment plant (7) did not hybridize with any of the isolates from the Plön treatment plant. It is possible that each treatment plant has its own hyphomicrobia, depending on the composition of incoming raw sewage and on the chosen treatment process.

The spatial distribution of some hybridization group members pointed to a more transient presence in some cases and indicated that there were preferred habitats in others. As shown in Table 4, members of HG 1 made up 9% of the isolates from the influent basin. Because their concentration in the activated sludge tank was 10^1 to 10^4 times higher, their presence in activated sludge cannot be explained only by migration from the influent but must have been due to an established resident population there. The same may apply to HG 26, although in this case, migration may have played a greater role (Table 4).

While HGs 1 to 3 and 25 to 28 occurred in significant numbers in the sludge tank and in the effluent, only HGs 25, 26, and 28 could be detected in the lake. Further studies may indicate whether HGs 25 and 26 could be employed as indicator organisms for sewage pollution.

The exclusive presence of HG 8 and 9 hyphomicrobia in the lake throughout the year was significant, but their seasonal frequency was not. The same applies to a lesser degree for HGs 10 to 20 and 22. Other lake isolates may have originated either from the sewage plant effluent (HGs 21, 25, 26, and 28) or from other sources (HGs 19, 20, and 22), as seemed to be especially the case with HG 22 hyphomicrobia, which hybridized with IFAM B-522, a typical soil isolate.

The most surprising findings of this investigation were the high number of genotypically distinct HGs and, for most of these groups, their very specific distribution patterns. Generally, this could be taken as an indication of an unexpectedly high number of new *Hyphomicrobium* species. In conclusion, this study demonstrated for the first time that the stages of a sewage treatment process contained genetically different hyphomicrobia: not necessarily those that arrived with the raw sewage, but different populations that appeared to be adapted to this specific treatment process.

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