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CHAPTER 10.4

The Genus *Herpetosiphon*

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The *Herpetosiphon* species are aerobic, chemoorganotrophic, filamentous bacteria that are Gram negative but do not have a typical Gram negative cell wall. The filaments are very long, unbranched, and multicellular, between 0.5 µm and 1.5 µm wide and usually 100 µm to more than 1200 µm long (Fig. 1). Short transparent sections ("sleeves"; Fig. 2) are seen at the ends of many filaments.

Sleeves are also found in related organisms, like *Chloroflexus*. Depending on the strain, the medium, and the age of the culture, the filaments may fragment into much shorter pieces and ultimately into single cells. The cells in the filaments are tightly attached to one another, and in living filaments, their boundaries can only be seen with a microscope of high resolution at a high magnification, by phase or interference contrast (Fig. 2). They become clearly recognizable if the filament has been fixed to the slide and stained. The cells have the same width as the filaments and measure 1.5–5 µm in length, rarely more. The filaments may perform slow gliding movements; thus, on suitable substrates, the colonies develop into large spreading swarms (Fig. 3). The surface of the swarm is usually felt-like. Long flame-like projections or protruding strands of curled filaments are seen at the edge (Fig. 3). Normally the colonies are colored in shades of yellow, orange, or brick red, but unpigmented strains, especially among *Herpetosiphon*-like strains, do occur (Lee, 2004). A film showing the movements of the filaments and the development of the colonies is available (Reichenbach et al., 1980).

Apparently, *Herpetosiphon* was first discussed in the scientific literature under the name "*Flexibacter giganteus*" (Soriano, 1945; Soriano, 1947). Soriano's isolation techniques, choice of habitats, and description of the filaments, the swarm colonies, and their color correspond to those of the cultures now called "*Herpetosiphon*." However, the first valid description of the genus *Herpetosiphon*, with one species, *H. aurantiacus* (ATCC 23779^T), was provided by Holt and Lewin (1968). The definition was based on three isolates of E. E. Jeffers obtained from the slime coat of a green alga, *Chara* sp., from Birch Lake in Minnesota. Later, a dispute arose about the presence of a sheath in *Herpetosiphon*, and the genus definition was slightly modified to exclude a sheath (Reichenbach and Golecki, 1975). Also, Reichenbach and Golecki (1975) proposed that the species name *aurantiacus* be abandoned in favor of the name *giganteus*, which antedates it. This change could have been done without danger of confusion since Soriano's strain is no longer available. However, since more than one *Herpetosiphon* species may exist, one could argue that Soriano's "*H. giganteus*" was different from *aurantiacus*. The possible existence of different species of *Herpetosiphon* was indicated by another study already during this period when a specimen was isolated from the walls of a sluice of the Neckar River near Heidelberg (Germany). This specimen showed a strong resemblance with *Herpetosiphon aurantiacus* but was not identified as such because of some minor differences in phenotypic characteristics (Brauss et al., 1969). Thus, it was decided to give up the name *giganteus* until more had been discovered about the taxonomy of the genus. In 1970, four new species, one from the vicinity of a hot spring (*H. geysericola* ATCC 23076^T) and three from marine habitats (*Herpetosiphon cohaerens* ATCC 23123^T, *Herpetosiphon nigricans* ATCC 23147^T and *Herpetosiphon persicus* ATCC 23167^T) were added to the genus (Lewin, 1970). However, all three marine species have recently been transferred to a new genus, *Lewinella*, in the phylum *Flexibacter-Bacteroides-Cytophaga* (Sly et al., 1998). Since 1998, over 30 new *Herpetosiphon* and *Herpetosiphon*-like isolates have been described from different environmental habitats, such as freshwater lake systems (Lake Constance, Germany; the strains were previously described as a new *Vitreoscilla* species, *V. proteolytica*; Gräf and Perschmann, 1970; Lee et al. (manuscript in preparation)), sewage plants (Trick and Lingens, 1984; Senghas and Lingens, 1985; Bradford et al., 1996; Kohno et al, 2002; Lee et al. (manuscript in preparation)), and different soil systems (Lee et al. (manuscript in

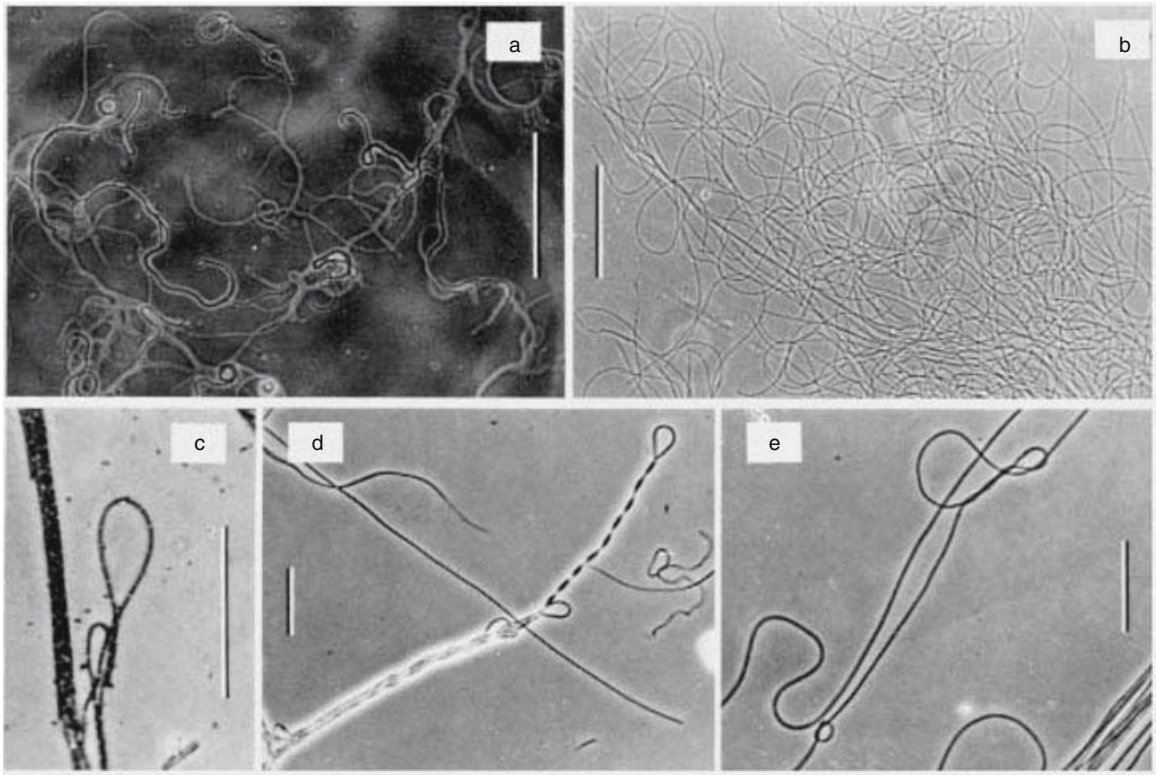

Fig. 1. Filaments of *Herpetosiphon aurantiacus* at low magnifications. a) Filaments with slime tracks in chamber culture. b) Filaments from a liquid culture, slide mount. c) Filaments stained with Loeffler's methylene blue; the individual cells of the filament are clearly visible. d) and e) Twisted filaments from a liquid culture. Zeiss Standard Microscope and Axiomat (d and e), in phase contrast. Bar = 100 μm (for a and b). Bar = 25 μm (for c, d and e).

preparation)). The G+C content of the DNA of about half of these isolates is 48–53 mol%, and the 16S rRNA gene similarity among these isolates is 96.4–99.5% (Sly et al., 1998; Lee et al. (manuscript in preparation)). However, the G+C content of the rest of the *Herpetosiphon*-like isolates, e.g., the strains of Senghas and Lingens (1985) and the strains Hp g6 (DSM 15710), Hp g16 (DSM 15736) and Hp g207 (DSM 15737), are higher (59–75%), which suggests that these isolates may represent novel genera among chloroflexi (Lee et al. (manuscript in preparation)).

A comparison of all present *Herpetosiphon* 16S rRNA gene sequences with all other available 16S rRNA gene sequences from culturable and as yet not cultivatable organisms clearly shows a phylogenetic relationship of *Herpetosiphon* to the third of the four suggested subphyla of the phylum *Chloroflexi*, previously non-green sulfur bacteria (Gibson et al., 1985; Woese et al., 1985; Hugenholtz et al., 1998a; Garrity and Holt, 2001; Lee et al. (manuscript in preparation)). The chloroflexi are well separated from all other bacteria and represent one of the main branches (a phylum) in the bacterial tree of descent. The similarity to other filamentous, gliding bacteria, such as *Vitreoscilla*, *Beggiatoa* and *Leucothrix*, which belong to the Betaproteobacteria and Gammaproteobacteria or the filamentous cyanobacteria, is significantly low (Reichenbach, 1981a; Reichenbach et al., 1986; Lee et al. (manuscript in preparation)). Interestingly, the relationship of *Herpetosiphon* to some chloroflexi members, e.g., *Chloroflexus*, is also reflected in other genes such as the elongation factor Tu or the ATP-synthase beta-subunit genes (Ludwig et al., 1993), as well as in the very unusual structure and composition of the cell walls of the two organisms. Both organisms contain a peptidoglycan in which *meso*-diaminopimelic acid is replaced by L-ornithine and to which a polysaccharide is covalently bound. In addition, both organisms lack a lipopolysaccharide (Jürgens et al., 1987; Jürgens et al., 1989).

Habitats

Typical *Herpetosiphon* strains are regularly found in freshwater, soil, and decaying organic matter (such as rotting wood, dung of herbivorous animals, and compost). *Herpetosiphon* appears to be rather common everywhere in aerobic environments, in the neutral pH range and

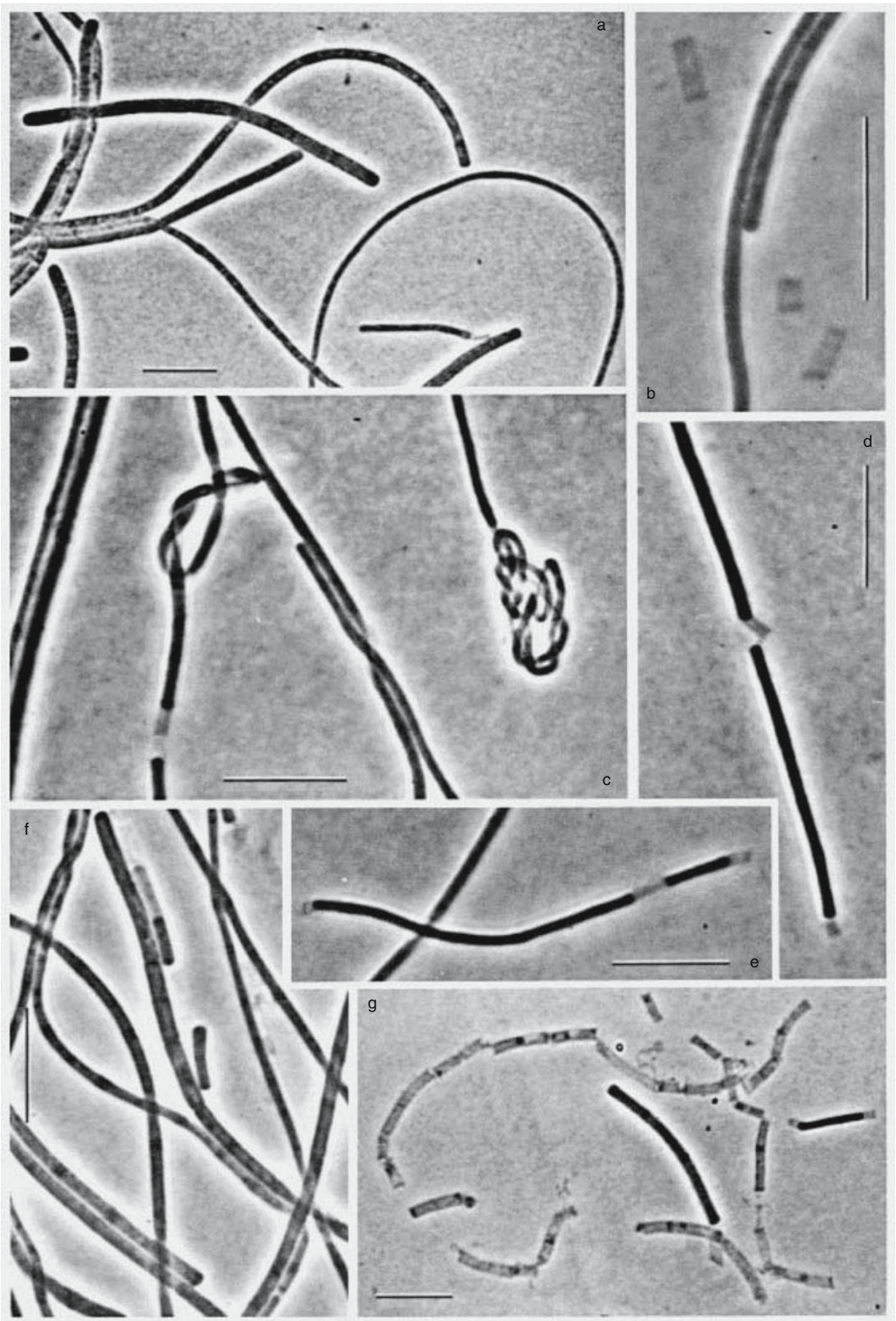
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Fig. 2. Filaments of the type strain of *Herpetosiphon aurantiacus* at high magnifications. a) Filaments from CY agar. Note the enormous variability of the filaments' diameter, which is especially pronounced on peptone media. Slight constrictions at the filaments' surface and dark bands in the interior indicate cell boundaries, which can be particularly clearly recognized in the thick filament in the upper left sector; dark dots seen in many cells may be mesosome-like membrane bodies. b) The translucent segments seen next to the filaments are empty cell-wall cylinders and correspond to the sleeves often found at the ends of the filaments; here, as in other figures of this table, parallel running filaments approach one another very closely, which seems to exclude the presence of a sheath of any significance. c) The knots in the filaments testify to their incredible flexibility; the filament on the left contains a necridium (a dead cell); from VY/2 agar. d) The filament has a short sleeve at the end and a necridium which is almost ready to snap. e) A short filament with sleeves at both ends and a necridium. f) In several filaments, cross-walls are recognizable; there is a one- and a two-celled segment, the latter with a living and a dead cell; from CY agar. g) Decaying filament fragmenting into cell-wall cylinders, each corresponding to one cell. Because the cylinders separate cleanly, there can be no sheath. All micrographs were taken with Zeiss Axiomat, in phase contrast. Bars = 10 µm.

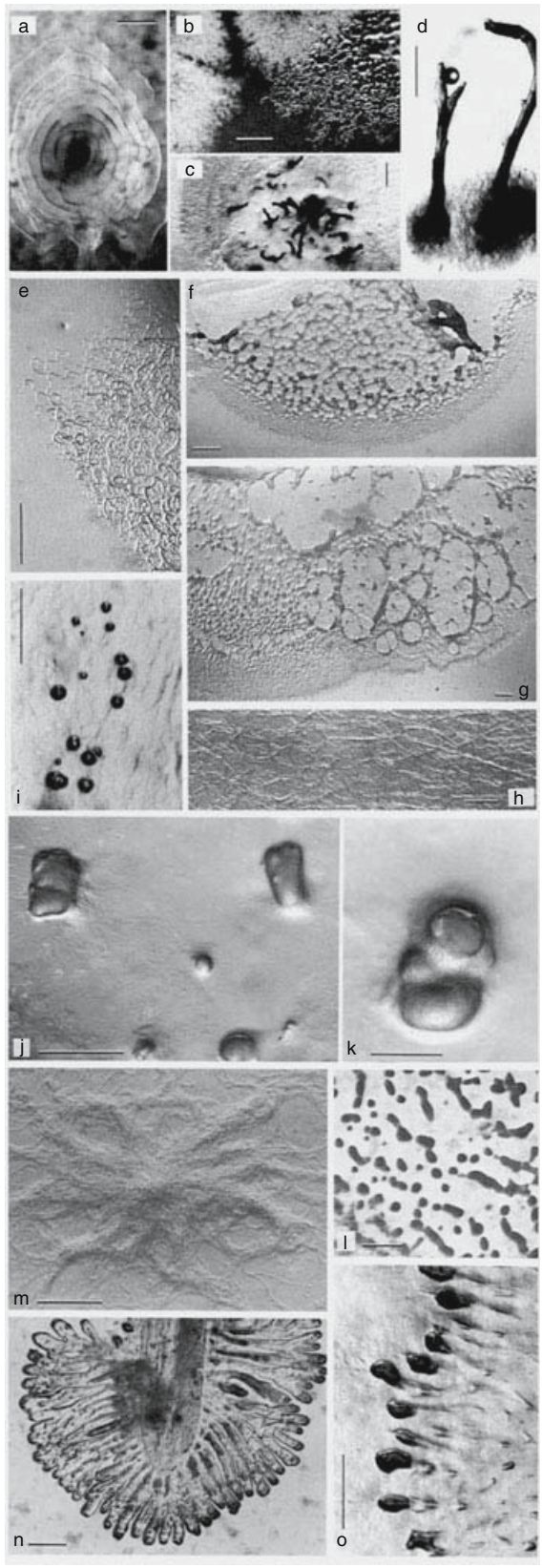

Fig. 3. Various swarm colony types of *Herpetosiphon aurantiacus*. The colonies of *Herpetosiphon* are very variable and occasionally show striking patterns. a) Swarm with a rhythmic growth pattern, on VY/2 agar. Bar = 1 mm. b) Swarm showing surface growth (right), as well as penetration into the agar (light areas). Bar = 1 mm. c) and d) Colonies with proboscis-like protuberances; c) survey picture; d) protuberances at higher magnification. Bar = 1 mm in both c) and d). e) Swarm growing from a streak of autoclaved *Escherichia coli* on water agar; the curly pattern is characteristic for this kind of culture; the cloudy areas in the lower part indicate penetration of the organism into the agar. Bar = 1 mm. f) and g) Large swarm on VY/2 agar; the swarm sheet tends to contract and peel off the agar surface, producing large holes surrounded by ridges; this growth pattern is very characteristic for *Herpetosiphon*. Bar = 1 mm in both f) and g). h) Delicately plicated surface of a swarm sheet on VY/2 agar; this is a relatively unusual pattern. Bar = 100 µm. i) to l) Knobs; they are sometimes produced in large numbers and arranged in patterns (i, l) that make their resemblance to myxobacterial fruiting bodies even more striking; also, their size is in the range of fruiting bodies. Bar = 1 mm in i) and l), 500 µm in j), and 200 µm in k). m) Microcolony showing its composition of long, coiling and interwoven filaments on CY agar. Bar = 100 µm. n) Swarm on a streak of autoclaved *E. coli* (center) on water agar spreading in an uncommon pattern of broad, tape-like tongues. Bar = 1 mm. o) Edge of a swarm growing on a streak of living *E. coli* on water agar; in contrast to the usual pattern on this medium, as shown in b) and e), here the swarm ends in a series of compact knobs with delicate, flame-like extensions (swarm center is to the right). Bar = 1 mm. Pictures made with Leitz Aristophot (a to g); Zeiss Axiomat (h, j, k and m); and Olympus SZH Stereo Microscope (i, l, n and o).

under mesophilic conditions. A study on the distribution of *Herpetosiphon* in the surface layers of Lake Constance (a large, deep, fairly oligotrophic lake in southwestern Germany) demonstrated the organism only in contaminated areas close to cities or at the mouths of rivers, while it was absent in clean stretches of water in the middle of the lake or along uninhabited and undisturbed shores (Gräf and Perschmann, 1970). This suggests that the *Herpetosiphon* population at the lake surface has its origin in the soil of the surrounding area and in contaminated waters running into the lake. The examples mentioned in the introduction show however that *Herpetosiphon* also thrives in the benthos of freshwater bodies. Therefore, its absence in the surface layers may be best explained by a fast sedimentation of the flocs of entangled filaments, which can stay in suspension only in vigorously agitated liquids.

Herpetosiphon and *Herpetosiphon*-like bacteria have also been observed and isolated from activated sludge of sewage plants on several occasions in different parts of the world (Salcher et al., 1982; Trick and Lingens, 1984; Senghas and Lingens, 1985; Bradford et al., 1996; Kohno et al., 2002; Björnsson et al., 2002; Lee et al. (manuscript in preparation)). *Herpetosiphon* may, however, have been observed on many more occasions in sewage material, but it has not been recognized as such. For instance, the organism described from a Dutch sewage plant as belonging to group III (Van Veen, 1973) may have been *Herpetosiphon* (strain Rz in Van Veen's Fig. 12, which shows the typical empty sleeves at the ends of the filaments). Interestingly, those bacteria were practically always present in activated sludge. The role of *Herpetosiphon* in sewage plants is unknown. However, if the filaments observed by Van Veen (1973) were indeed *Herpetosiphon*, it may represent one of the many biological agents for the "bulking" problems in sewage plants (for a review, see Eikelboom [1983] or Jenkins et al. [2003]). On the other hand, recently Björnsson et al. (2002) and later Lee et al. (manuscript in preparation) speculated that filaments belonging to the chloroflexi and, thus among others, *Herpetosiphon*, may be one of the crucial agents for the important backbone structure of activated sludge flocs, thus suggesting a positive role for at least some chloroflexi filaments in sewage plants.

One species, *H. geysericola* (ATCC 23076^T), has been described from the surroundings of a hot spring (Lewin, 1969a; Lewin, 1970). It is represented by just one strain, which in addition appears to be thermotolerant rather than thermophilic (the literature is completely mute about that important point; but the American Type Culture Collection [ATCC] catalog lists a growth temperature of 30°C for the strain). The environment from which *H. geysericola* was isolated is of interest insofar as this is the typical habitat of the related thermophilic phototroph *Chloroflexus*. In a study in a Yellowstone hot spring (Yellowstone National Park, United States), it was shown that the bacterial mat contained two other types of filamentous bacteria besides *Chloroflexus* (Tayne et al., 1987). Unfortunately, the antiserum used to identify *Chloroflexus* was not tested for crossreactions to *Herpetosiphon*. It is thus still unknown whether *Herpetosiphon* may be more common in such environments than has so far been assumed. Even though several molecular surveys on the microbial population in different hot springs and microbial mats have not retrieved any *Herpetosiphon*-related 16S rRNA gene sequences (e.g., Pierson et al., 1994; Hugenholtz et al., 1998b; Boomer et al., 2002; Nübel et al., 2002), this is still not a final proof for their nonexistence in these systems in particular since many of these studies used molecular markers specific only for phototrophic members of chloroflexi (Pierson et al., 1994; Boomer et al., 2002; Nübel et al., 2002). Lee et al. (manuscript in preparation) showed that although it was possible to isolate *Herpetosiphon* from activated sludge as well as observe it *in situ* by microscopy using *Herpetosiphon*-specific oligonucleotide probes, no *Herpetosiphon* affiliated clones could be retrieved from a 16S rRNA clone library based on general *Bacteria* polymerase chain reaction (PCR) primers, although 10% of the obtained clones were indeed affiliated to other as yet not cultivatable members among the chloroflexi. This suggests that *Herpetosiphon* may not be so easily retrieved from the environment in 16S rRNA clone library surveys on the basis of general PCR primers.

While *Herpetosiphon* obviously may be a common inhabitant of freshwater, it is equally at home in nonaquatic environments. One of us has isolated (as a byproduct of myxobacteria isolation) in the course of 20 years more than 400 *Herpetosiphon*-like strains from soil samples, dung pellets, rotting wood, and similar materials collected all over the world in various climate zones, including decidedly hostile, dry, and hot environments, like semi-deserts in Tunisia and Arizona, steppe habitats in Mediterranean countries, the Canary Islands, and gypsum hills on Cyprus (H. Reichenbach, unpublished observations). Twenty-eight of these isolates have been further examined. Ten of these isolates were related to *H. aurantiacus* (16S rRNA gene similarity >97%), whereas the rest most likely represent novel genera among chloroflexi (Lee et al. (manuscript in preparation)). It seems that the diversity of *Herpetosiphon* is larger in soil than in aquatic environments, since all isolates obtained so far from freshwater environments or sewage plants (with the exception of some iso-

lates of Senghas and Lingens [1985], which are unfortunately no longer available for further comparison) are closely related to *H. aurantiacus*, whereas some of the soil isolates represent most likely novel genera (Lee et al. (manuscript in preparation)).

Isolation

Herpetosiphon may be isolated from any of the sources mentioned in the section Habitats in this Chapter. The terrestrial strains usually survive desiccation so that dry samples can also be used. One of us was able to obtain *Herpetosiphon* from rotting wood after eight years storage, from filter-paper preserves of contaminated myxobacteria after six years, and from soil after two years of storage in the dry state at room temperature (H. Reichenbach, unpublished observations). It has been noted that strains from sewage plants may also be extremely resistant to desiccation (Trick and Lingens, 1984).

No specific enrichment techniques are known. The strains on which the definition of the genus is based were isolated by making a single streak from the slimy coat of *Chara* on a plate with 0.3% peptonized milk (Difco) and 1.5% agar. The culture was incubated at room temperature. Evidently, this relatively rich medium can be used only if the sample contains few other microorganisms (Holt and Lewin, 1968). Therefore, for a more successful isolation, use is made of the ability of the organism to glide away from the inoculum and to produce a typical swarm pattern. To elicit gliding and to suppress excessive growth of contaminants, media very low in organic constituents are recommended. The simplest medium is plain water agar, to which cycloheximide may be added to prevent the development of fungi:

WAT Agar

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Dissolve the ingredients in distilled water and adjust the pH to 7.2 with KOH. The pH adjustment is much easier if HEPES (20 mM) is added as a buffer. For crude cultures, use WCX agar, which is WAT agar plus a filter-sterilized stock solution of cycloheximide (25 μg/ml of medium).

Several small samples of soil or other sources of inoculum (size of a lentil) are placed on the surface of the dry plate, a few cm from each other, and the culture is incubated at 30°C. After 2–20 days, the typical swarm colonies with long flares and coils of entangled filaments at the edge may be observed (Fig. 3). The very delicate pattern can only be seen if an oblique light beam produced by a tiltable mirror is applied from below. We have had success with WAT agar plates with three thin, parallel streaks of living *Escherichia coli*, each of which is inoculated at one end. *Herpetosiphon* grows in a spreading fashion within the streaks, usually without lysing the *E. coli* cells, and often only appears along the edges of the streaks in the form of long flame-like structures or a loose pattern of separate, coiling strands of filaments. Sometimes, large and more or less dense swarm sheets are produced that spread over much of the plate. An especially high yield of *Herpetosiphon* can sometimes be obtained if such cultures are incubated at 38°C.

For the isolation of planktonic *Herpetosiphon* in freshwater, between 0.5 and 1 liter of lake or river water is passed through a sterile membrane filter (pore size, 0.4 μm). The filter is then cut into pieces, and the segments are placed on a low-nutrient agar medium. In the original study, rabbit dung agar was used but any other lean medium would certainly also do.

Rabbit Dung Agar (Gräf and Perschmann, 1970)

Boil dry dung pellets from wild rabbits (20 g) in 1 liter of distilled water for 20 min. Add 15 g of agar to 1 liter of filtrate. Adjust the pH to 7.2.

The cultures are incubated at 33°C. After about 6 days, swarm colonies begin to spread from the edges of the filter.

Different procedures have been applied for the isolation of *Herpetosiphon* from activated sludge. One procedure consists of streaking a drop of the sludge sample on agar plates or embedding it in agar itself (Trick and Lingens, 1984; Senghas and Lingens, 1985). Another procedure is based on micromanipulation of filaments in the activated sludge (Bradford et al., 1996). Different media have been used, ranging from complex but rather nutrient-poor media, such as BG-11 agar (originally designed for the isolation of cyanobacteria), I agar (Trick and Lingens, 1984; Senghas and Lingens, 1985) or R2A (Bradford et al., 1996) to sludge-based media (Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). As sewage organisms are notoriously fastidious and adapted to very low nutrient levels, we have listed below all the media used; whether they are really required or could be replaced by simpler recipes has not been determined. The cultures are incubated at 25°C and examined with a phase contrast microscope over several weeks (Salcher et al., 1982; Bradford et al., 1996). Pure cultures can be obtained by transferring filaments from the advancing swarm edge to the same media again, or, as soon as the culture is sufficiently pure, to richer media (see the section Cultivation in this Chapter). We often have good results with transfers to streaks of autoclaved *E. coli* on water agar, although some *Herpetosiphon* strains do

not grow on autoclaved bacteria (see the section Cultivation in this Chapter). As the organism has to be carefully removed from the contaminated plate, transfers are made best by cutting out a piece of the swarm edge on a small agar block using a drawn-out glass rod or, more conveniently, a fine steel injection needle (e.g., on a 1-ml disposable syringe). The procedure is repeated until the strain is pure. Two properties of *Herpetosiphon* may substantially speed up purification: We have found that all *Herpetosiphon* strains tested so far grew at 38°C and are resistant to 250 mg of kanamycin sulfate per liter.

BG-11 Agar (Stanier et al., 1971)

$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA(Na_2-Mg salt)	0.001 g
Na_2CO_3	0.02 g
Trace element solution A5	1 ml
Agar	15 g
Distilled water	1 liter

The trace element stock solution A5 contains per liter: H_3BO_3, 2.8 g; $MnCl_2 \cdot 4H_2O$, 1.8 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g; $CuSO_4 \cdot 5H_2O$, 0.08 g; and $Co(NO_3)_2 \cdot 6H_2O$, 0.05 g.

I Agar (modified from Van Veen, 1973; Salcher et al., 1982)

Glucose	0.15 g
$(NH_4)_2SO_4$	0.5 g
$Ca(NO_3)_2$	0.01 g
K_2HPO_4	0.05 g
$MgSO_4 \cdot 7H_2O$	0.05 g
KCl	0.05 g
$CaCO_3$	0.1 g
Agar	15 g
Distilled water	1 liter

After autoclaving, add 10 ml of a filter-sterilized vitamin solution. The vitamin solution contains per liter: Ca pantothenate, 10 mg; nicotinic acid, 10 mg; biotin, 0.5 mg; cyanocobalamin, 0.5 mg; folic acid, 0.5 mg; pyridoxine·HCl, 10 mg; *p*-aminobenzoic acid, 10 mg; thiamine pyrophosphate, 10 mg; thiamine, 10 mg; inositol, 10 mg; and riboflavin, 10 mg.

R2A Agar (Reasoner and Geldreich, 1985)

Enzymatic digest of casein	0.25 g
Enzymatic digest of animal tissue	0.25 g
Acid hydrolysate of casein	0.5 g
Yeast extract	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
$KH_2 PO_4$	0.3 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Sodium pyruvate	0.3 g
Agar	15 g
Distilled water	1 liter

Adjust pH to 7.2.

Sludge-based Media (Fuhs and Chen, 1975)

CH_3COOH-Na	5 g
$(NH_4)_2SO_4$	2 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KH_2PO_4	0.25 g
$CaCl_2 \cdot 2H_2O$	0.2 g
Agar	15 g
Mixed liquor (sewage/sludge)	200 ml
Distilled water	800 ml

Adjust pH to 7.0.

VY/2 Agar

Bakers' yeast (fresh weight)	0.5%
$CaCl_2 \cdot 2H_2O$	0.1%
Cyanocobalamin	0.5 mg/liter
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.2.

CY Agar

Casitone (Difco)	0.3%
Yeast extract	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.2.

CYCAS Agar

Casitone (Difco)	0.3%
Yeast extract	0.3%
$CaCl_2 \cdot 2H_2O$	0.1%
Cellulose powder	0.1%
Soluble starch	0.1%
Acetate	0.1%
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.2.

ATCC Medium 810 (Myxo 810 Medium)

Skim milk powder	0.5%
Yeast extract	0.05%
Agar	1.5%

Dissolve ingredients in distilled water. Do not adjust the pH.

EC Medium (Enriched Cytophaga Medium; Pate and Chang, 1979)

Tryptone (Difco)	0.2%
Yeast extract (Difco)	0.05%
Na acetate	0.02%
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.5.

Escherichia coli Overlay Agar

Suspend the growth of four culture plates of *E. coli* (on any suitable medium) in 100 ml of water agar (WAT agar; see the section Isolation in this Chapter). Autoclave the suspension and pour as a thin layer on top of water agar plates.

Medium 2 (Lewin and Lounsbery, 1969b)

Casamino acids	0.1%
Na glycerophosphate	0.01%
Tris buffer	0.1%

KNO3	0.01%
$CaCl_2 \cdot 2H_2O$	0.01%
$MgSO_4 \cdot 7H_2O$	0.01%
Thiamine	1 mg/liter
Cyanocobalamin	1 mg/liter
Agar	1%
Glucose	0.1%

Dissolve all ingredients except for glucose in distilled water and adjust to pH 7.5. Add separately autoclaved glucose after autoclaving the solution of the other ingredients.

Most of the cultivable strains also grow in liquid media, in shake flasks, and in fermentors. A good medium for many strains is HP74 liquid medium. Other strains grow equally well or better in peptone-containing media, e.g., in CAS liquid medium, R2A liquid medium, or MDl liquid medium.

HP74 Liquid Medium

Na glutamate	1%
Yeast extract (Difco)	0.2%
$MgSO_4 \cdot 7H_2O$	0.2%
Glucose	1%

Autoclave the glucose separately as a 20% stock solution. Add 20 ml per liter of separately autoclaved phosphate buffer (50 mM, pH 6.5) to give 1 mM.

CAS Liquid Medium

Casitone (Difco)	1%
$MgSO_4 \cdot 7H_2O$	0.1%

The pH should be 6.8; do not adjust.

MDl Liquid Medium

Casitone (Difco)	0.3%
$CaCl_2 \cdot 2H_2O$	0.07%
$MgSO_4 \cdot 7H_2O$	0.2%
Cyanocobalamin	0.5 mg/liter

Add trace element solution (see below) after autoclaving the MD1 medium. The pH should be 6.8; do not adjust. If Casitone (Difco) is replaced by other enzymatically digested casein peptones, adjust pH to about 7.

Trace Element Solution

$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2$	20 mg
$CuSO_4$	10 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$ZnCl_2$	20 mg
LiCl	5 mg
$SnCl_2 \cdot 2H_2O$	5 mg
H_3BO_3	10 mg
KBr	20 mg
KI	20 mg
EDTA, Na-Fe_3^+ salt (trihydrate)	8 g
Water	1 liter

Sterilize by filtration and add 1 ml per liter of medium. The filter-sterilized solution is stable for months at room temperature because of its high EDTA content.

Cultivation

In general, *Herpetosiphon* prefers low nutrient levels. Thus, one of our strains, Hp g175, produced good growth over six transfers on plain water agar, obviously living off minute quantities of contaminating material in the agar but without visibly attacking the agar itself. Rich media, like nutrient agar or nutrient broth, are often not suitable at all. About one out of three strains isolated from soil on streaks of living *E. coli* can be cultivated without difficulty on complex media, such as VY/2 agar, CY agar, CYCAS agar, milk-based agar media (e.g., ATCC medium 810) or R2A agar (see the section Isolation in this Chapter). Isolates obtained from freshwater or sewage systems may also be cultivated on these media and in addition on EC and I agar media (see section Isolation in this Chapter). *Herpetosiphon geysericola* (ATCC 230767^T) was cultivated on medium 2 (Lewin, 1970). This medium was used as a standard medium for the cultivation of many different organisms; thus, not every component may be required by *H. geysericola* (e.g., thiamine). *Herpetosiphon geysericola* however also grows on all of the media mentioned above.

Many strains produce heavy growth and often spread within a few days over the entire plate. Some strains tend to penetrate the agar (Fig. 3). The yeast in VY/2 agar may or may not be lysed. However, a significant number of the soil isolates obtained from water agar media with streaks of living *E. coli* cannot be further cultivated on other media. Only a few of them respond favorably to a reduction of the nutrient concentration and can be grown on media like VY/5 agar (such as VY/2 agar but with yeast concentration reduced to 0.2%). Others grow more or less poorly on *E. coli* overlay agar, but most of them can barely be kept alive on streaks of autoclaved *E. coli* on water agar. Some of these strains survive only on living bacteria, presumably because they require some labile growth factor. The alternative explanation (i.e., *E. coli* removes some inhibiting component) seems less likely because the filaments often creep far away from the *E. coli* streak. All our efforts to identify growth factors have been unsuccessful, although many possibilities have been tested (different vitamins, sugars, and amino acids; plant oil; chitin, catalase, and cell fractions of *E. coli* and of cultivable *Herpetosiphon* strains; anaerobic and microaerophilic conditions; different agar concentrations, temperatures, and pH values; illumination). Recently, we found that four of these fastidious strains, Hp g122 (DSM 15874); Hp g124 (DSM 15875); Hp g150 (DSM 15876), and Hp g174 (DSM 15877) were only distantly related to the type strain *Herpetosiphon aurantiacus* ATCC

23779^T (ca. 80% 16S rRNA gene sequence similarity), which strongly indicates that they represent a new genus among chloroflexi (Lee et al. (manuscript in preparation)).

In agitated cultures the organisms may grow as homogeneous suspensions, but often the filaments stick together to form flakes or tight spherules. One of our strains, Hp a2 (DSM 589), a member of the *H. aurantiacus* species group, forms flakes when inoculated into HP74 liquid medium; the cultures become completely uniform after a few days, but the organism forms flakes again upon transfer into fresh medium. Growth is not particularly fast: The doubling time is in the range of 15–20 h, and the cultures do not enter the stationary phase before 4–6 days of cultivation (30°C, HP74 liquid medium, shake flasks). Wet-weight yields are in the order of 1–3 g/liter in shake flasks and 2–4.5 g/liter in fermentors (HP74 liquid medium). The dry weight amounts to roughly 20% of the wet cell mass. Trick and Lingens (1984) and Senghas and Lingens (1985) obtained similar values (doubling time 20–25 h; cell yield after 5–6 days, 2–3.5 g wet weight per liter and 0.5–1 g dry weight per liter) with their sewage isolates when cultivating them on EC medium at 25°C, their temperature optimum.

All known strains are strictly aerobic. The temperature range differs with different strains. In general, the freshwater strains seem to prefer lower temperatures, around 25°C, while the soil and some freshwater strains grow very well at 30°C and above. All *Herpetosiphon* and *Herpetosiphon*-like soil strains tested by us grew at 38°C, and some *Herpetosiphon*-like strains still grew at 45°C (e.g., Hp g207, DSM 15737; Lee et al. (manuscript in preparation)). The sewage strains showed a temperature minimum at 15°C, a maximum at 37°C, and an optimum at 25°C (Trick and Senghas, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). *Herpetosiphon geysericola* comes from the vicinity of a hot spring but can be cultivated at 30°C (see the section Habitats in this Chapter). The optimal pH range is 7–7.5, although pH values of 8–9 seem occasionally to be tolerated.

Preservation

Most *Herpetosiphon* strains survive on agar media at 30°C or room temperature for up to 3 months. Slant cultures stored at 4°C have been successfully subcultured after 14 months. However, some *Herpetosiphon*-like strains, such as Hp g6 (DSM 15710), Hp g16 (DSM 15736) and Hp g207 (DSM 15737), which represent more likely novel genera of chloroflexi rather than *Herpetosiphon*, are more sensitive to long-term storage at room temperature or at 4°C. These, as well as stock cultures, should be transferred every 3–4 weeks (Lee et al. (manuscript in preparation)).

Herpetosiphon strains may be preserved by any of the standard procedures. A convenient and reliable method is storage at –80°C. The bacteria are suspended in 1 ml of CAS or HP74 liquid medium and simply put into the deep freeze. Thawing should be fast, best accomplished by immersing the tube in cold water, and transfers to a suitable growth medium have to be made immediately after thawing. We have been able to reactivate such preserved cultures with no problems after 8 years of storage, the longest period tested. Alternatively, the bacteria can be frozen in liquid nitrogen. In one study, the filaments were suspended in growth medium (medium 2 as above, without agar), supplemented with 10% of either glycerol or dimethyl sulfoxide (Sanfilippo and Lewin, 1970). All strains survived freezing and thawing with both additives. Only the glycerol preserves were tested again after 1 year, and all strains were still viable. When stored in growth medium without additives at –22°C, three out of four strains survived for 21 weeks (the longest period tested) and one for 6 weeks. Strains dried in skim milk could always be reactivated after storage for up to 12 years (unpublished data). We do not freeze-dry the organism itself but only the skim milk: A few drops of a thick suspension of *Herpetosiphon* filaments, taken from a young plate culture, are added to a plug of freeze-dried skim milk in an ampoule. The plug absorbs the liquid without liquefying itself. The ampoule is then dried at room temperature in a desiccator on a vacuum pump for several hours. After filling the desiccator with nitrogen gas, the ampoules are sealed.

Herpetosiphon strains are currently available from six different cultures collections: ATCC (the American Type Culture Collection, Rockville, USA), ACM (the Microbial Culture Collection of the University of Queensland, Brisbane, Australia), CCUG (Culture Collection University of Göteborg, Sweden), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), NCIMB (the National Collection of Industrial Food and Marine Bacteria, University of Aberdeen, Scotland), and TISTR (the Culture Collection of Bangkok, MIRCEN, Thailand). The largest collection is deposited at DSMZ, encompassing the "Hans Reichenbach Collection" of 460 *Herpetosiphon* and *Herpetosiphon*-like strains; however, only around 30 of these strains have been further characterized (Lee et al. (manuscript in preparation)).

Morphological Characteristics

The filamentous morphology and the "sleeves" of *Herpetosiphon* are rather distinct so that the bacterium can readily be recognized under the microscope. However, some of the other gliding filamentous genera among chloroflexi, e.g., *Chloroflexus*, *Heliothrix* and *Roseiflexus*, share certain morphological features with *Herpetosiphon* (Garrity and Holt, 2001; Lee et al. (manuscript in preparation)). Fortunately, since most of these latter mentioned filaments are mainly thermophilic and facultative phototrophic, a certain distinction may still be possible by knowing the habitat, the physiology or the pigmentation of the organism. Nevertheless, for a final identification, a molecular method, such as 16S rRNA gene analysis or fluorescence in situ hybridization (FISH) using oligonucleotide probes is strongly recommended (for a review of oligonucleotide probes used for detecting different members of *chloroflexi*, see Björnsson et al. [2002] and Lee et al. (manuscript in preparation)).

General Morphology

The morphology of the two acknowledged species (*aurantiacus* and *geysericola*) of *Herpetosiphon* is rather similar. *Herpetosiphon* forms extremely long, unbranched, multicellular filaments of uniform diameter (Fig. 1). The filaments often measure 100 μm to more than 1200 μm in length. The cells in a filament cohere tenaciously so that the filament does not break easily. Unconstrained filaments in a liquid medium appear straight and stiff, but in fact they are extremely flexible and can sharply bend back and twist around themselves in a rope-like fashion or intertwine into plait-like masses (Fig. 1). The width of the filaments varies with different strains (0.5–1.7 μm), mostly between 0.7 μm and 1.2 μm. There is also considerable variation within the same culture (Fig. 2). For instance, we have measured filament diameters between 0.7 μm and 1.6 μm in an 11-day-old culture of the type strain of *H. aurantiacus* on CY agar. In older cultures or under unfavorable conditions, the filaments often swell substantially and may become completely irregular in outline. Furthermore, the filaments are often locally inflated and form large, spherical structures, or "bulbs," with a diameter of 2.5–3.5 μm (Reichenbach and Golecki, 1975; Trick and Lingens, 1984; Fig. 4). Under the electron microscope a membrane body at the end of a septum-like stalk can always

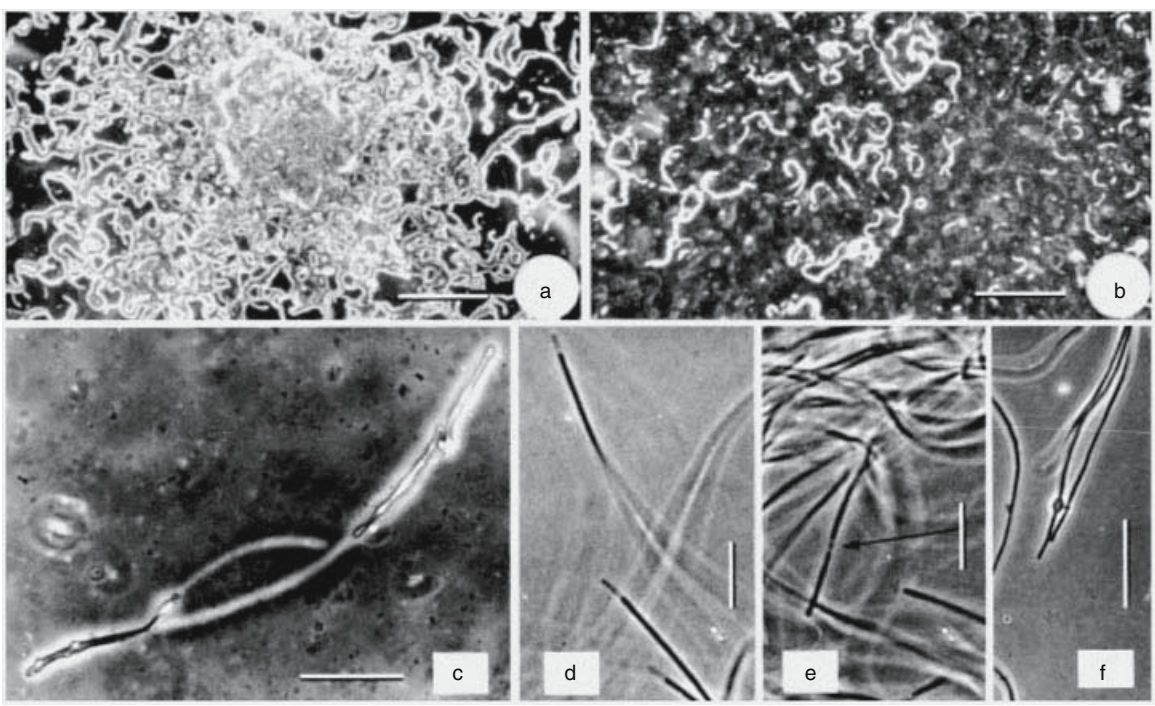

Fig. 4. Special structures of *Herpetosiphon aurantiacus*. (a to c) Desiccation-resistant filaments in a chamber culture. a) and b) Survey pictures; dark field; Bars = 200 μm: a) 9 days old, with the filaments still essentially intact; b) 32 days old, with only remnants of the filaments left. Bar = 200 μm. c) At high magnification, the remaining segments of the old filaments appear irregular in outline and optically refractile; there are conspicuous slime tracks. Bar = 30 μm. d) The empty sleeves, which are visible here at the ends of many filaments, are easy to recognize, even at a relatively low magnification, and thus help to quickly identify an isolate as a *Herpetosiphon*. Bar = 10 μm. e) A necridium (arrow). Bar = 10 μm. f) Bulbs. Bar = 20 μm. Zeiss Standard Microscope (a to c). Zeiss Axiomat (d to f); (c to f) in phase contrast.

be seen within the bulb. Those bulbs seem to be a degeneration phenomenon, and outgrowth or further development has never been observed from them. In old cultures, the filaments also tend to become shorter, especially on peptone-containing media, simply because more and more cells die and the filaments snap at such sites. The fragments can be as short as one cell. In old cultures, particularly on agar, short, optically refractile, irregular filaments are often seen (Fig. 4). As those are the only structures left in old, drying, but still viable cultures, they appear to be desiccation-resistant resting forms. However, their germination has not been directly observed so far.

The filaments consist of cylindrical cells that have the same diameter as the filament, and they are so closely attached to one another that only exceptionally shallow notches can be seen at the surface. Under a microscope with a high resolution using phase or interference contrast, the cells can sometimes be seen in living filaments (Fig. 2). The septa become more conspicuous in older cultures, particularly when the organism is grown on a peptone-rich medium, like CY agar. In such filaments the intracellular membrane bodies (see below) can often also be recognized (Fig. 2). The cells become clearly distinguishable if the filaments are first dried to the slide and then stained, e.g., with Loeffler's methylene blue, crystal violet, or alcoholic fuchsin (rosaniline). The lengths of the cells vary even within one filament, which suggests that the cells do not divide synchronously. The cell length may also vary with the culture medium, the culture age, and the strain. With many strains, cell lengths between 1.5 μm and 4.5 μm have been measured. The type strain of the genus appears to have especially long cells, measuring between 3 μm and 7 μm and sometimes 10 μm.

In wet mounts, *Herpetosiphon* filaments often can be seen to bend slowly and sometimes to slowly oscillate back and forth. In contact with a suitable interface, i.e., one to which the organism can stick, like a glass or an agar surface, the filaments glide slowly and, during this movement, deposit characteristic slime tracks (Fig. 1). Gliding filaments often bend laterally to form hairpin-like loops (Fig. 1). In liquid media, the filaments apparently move along themselves. They may coil around themselves or one another and produce all kinds of loops and knots (Figs. 1 and 2).

Sleeves or Sheathes

A most characteristic feature of *Herpetosiphon* is the presence at the ends of many filaments of transparent sections, which we call sleeves (Fig. 2). The sleeves have the same diameter as the filaments (1.4–5.5 μm) and are usually 1.5–3.5 μm long (the measurements are those for strain Hp a2, DSM 589, member of the *H. aurantiacus* group). The role of those sleeves is still controversial. Some investigators have regarded them as an indication of a sheath (Holt and Lewin, 1968; Skerman et al., 1977) or at least of a microsheath (Trick and Lingens, 1984). We prefer a different explanation (Reichenbach and Golecki, 1975): The filaments appear to multiply by breaking at the sites of necridia, i.e., dead cells along the filament. Such necridia can occasionally be observed in young cultures, and their number increases substantially with the age of the culture (Fig. 2). Their length (1.5–3.7 μm) is in the size range of ordinary cells. Trichome division by breakage at necridial cells is well known from other bacteria and has been described, e.g., for *Oscillatoria* (Lamont, 1969) and *Beggiatoa* (Strohl and Larkin, 1978). When a filament snaps, the empty cell wall cylinder of the necridium remains attached to the end of one daughter filament and gives rise to a sleeve. In strain Hp a2 (DSM 589), the outer wall seems to rupture at one of the ends of the necridium (near the cross-wall rather than across its middle), for the shortest and the longest sleeves correspond exactly to the lengths of the shortest and the longest cells. This would also explain why only some of the filaments' ends bear a sleeve, although it seems that the sleeves may also be shed; often empty sleeves can be seen lying in between the filaments. With the type strain of *H. aurantiacus*, on the other hand, the sleeves are exceptionally short, with a size distribution corresponding approximately to one-half that of the cell length, so that here the necridia seem to break preferentially in their center.

There are at least six more arguments against the presence of a sheath: 1) The filament glides as a whole, with its empty end-pieces in position, which would seem unlikely if the cells were enclosed in a sheath. 2) There is no movement of cells within the filament, e.g., at the site of a necridium, nor a release of cells at the ends of the filament. This was observed by Holt and Lewin (1968) and also by the present authors. But the literature is not in complete agreement on these points. Movement of a filament within its sheath has been described and verified by time-lapse photography (Skerman et al., 1977); however, it is our impression that what was described as a sheath may really have been a slime trail. Also, it was stated that single, gliding cells were released from the filaments (Brauss et al., 1969), but in this case what was seen may simply have been short fragments that broke off the ends of the filament. 3) If the sleeve were a piece of a sheath, it would be difficult to understand why, as a rule, it does not exceed the length

of a cell. 4) If a sheath were present, one could expect an occasional false branching of the filaments. 5) When a whole filament dies and decays, it falls apart into cell-sized pieces (Fig. 2). Obviously, there is no sheath to hold them together. 6) We have never been able to demonstrate anything resembling a real sheath under the electron microscope, with specimens prepared by freeze-etching, negative contrast, metal shadowing, or thin-sectioning after several different fixation protocols (Reichenbach and Golecki, 1975; see also Gräf and Perschmann, 1970). This is also true for the type strain (Fig. 5). The thin, tube-like structure composed of fibrils, which has been described by several investigators and has been interpreted as a microsheath (Skerman et al., 1977; Senghas and Lingens, 1985; Holt, 1989), probably has a different explanation.

How can these differences of opinion about the existence of a sheath in *Herpetosiphon* be reconciled? Differences among strains can probably be ruled out, for we included the type strain in our studies and found no significant divergence from strain Hp a2 (DSM 589). A sheath is a morphologically distinct structure, a hollow cylinder with a discrete outer boundary, excreted by the cells and not covalently bound to their surface. Under the light microscope, a sheath should be clearly recognizable, e.g., after negative staining with nigrosin or India ink. Under the electron microscope, a sheath may be difficult to visualize in thin sections because of poor contrast with the usual techniques, but with negative contrast or metal shadowing, it should be seen. Like all gliding bacteria, *Herpetosiphon* excretes slime, and slime fibrils can always be observed in the neighborhood of the trichomes, but this material is more or less diffuse. Under certain conditions this slime may condense, perhaps as a consequence of dehydration during fixation, and then it may appear as a dense tube. This is probably what has been taken as evidence for a sheath by various investigators (Skerman et al., 1977; Senghas and Lingens, 1985; Holt, 1989). Typically in all those cases cited, a wide gap opens between the filament and the thin, dense tube. Senghas and Lingens (1985) made efforts to demonstrate a sheath in their *Herpetosiphon*-like strains by first lysing the cells in the filament with lysozyme and then staining the remains with crystal violet. In that case, indeed, a long thin tube-like structure could be produced. However, this is still not an unequivocal proof of a sheath, for the technique requires as a prerequisite for reliable results that the organism has a normal bacterial cell wall.

Electron microscopy of thin sections of Hp a2 (DSM 589) reveals, however, that *Herpetosiphon* has an unusual cell wall structure (Reichenbach and Golecki, 1975). Outside the cytoplasmic membrane there is a well-defined peptidoglycan layer, 4–6 nm thick, but no outer membrane can be resolved. In fact, that membrane seems to be absent. Rather there is a thin additional layer, 21–25 nm thick, granular in thin sections, fibrillar in freeze-etch preparations, on top of the peptidoglycan. This layer follows the peptidoglycan into the shallow notches between adjoining cells but not into the cross-septa where only the peptidoglycan layer can be distinguished. We have never found any other defined layer outside the fibrillar layer, and in particular, have found no layer running smoothly across the cell junctions, as would be expected for a sheath. This peculiar wall structure is corroborated by chemical analyses of the cell envelope (see below) and may be directly connected with the sheath problem. Apparently, the additional layer is considerably more resistant to decomposition than the usual lipopolysaccharide-containing outer membrane, and it may give the peptidoglycan, to which it seems to be covalently bound, a higher stability. Within the cells there are extensive intracellular membrane systems, some of which originate at the cross-septa, others from the cytoplasmic membrane along the longitudinal walls. They become especially conspicuous when the negative-contrast technique is applied. As mentioned above, under certain conditions they can even be seen in the light microscope. Recent studies on the cell envelopes of Hp a2 (DSM 589) have revealed further interesting features, showing that ion-permeable channels, formed by a 45-k-Da protein, were present in the cell wall. This suggests that this strain has a permeability barrier on its surface similar to the outer membrane of Gram-negative bacteria, a feature which it shares with certain other Gram positive organisms, such as *Mycobacterium chelonae*, *Corynebacterium glutamicum*, *Streptomyces griseus* and *Micromonospora purpurea* (Harwardt et al., 2004).

Colony Morphology

The colonies, or swarms, usually spread quickly over agar surfaces and may completely cover a culture plate within a few days. *Herpetosiphon*-like strains, such as Hp g16 (DSM 15736) and Hp g207 (DSM 15737) grow more slowly, and may need up to 2–3 weeks to produce a significant spreading growth, although the plate is rarely completely covered (Lee et al. (manuscript in preparation)). On poor media, the migrating filaments remain more or less separate and appear in the form of characteristic curls and whirls (Fig. 3). Such colonies may become extremely delicate and are barely recognizable even if oblique illumination is applied. On media that allow good growth, dense and tough swarm sheets arise, with

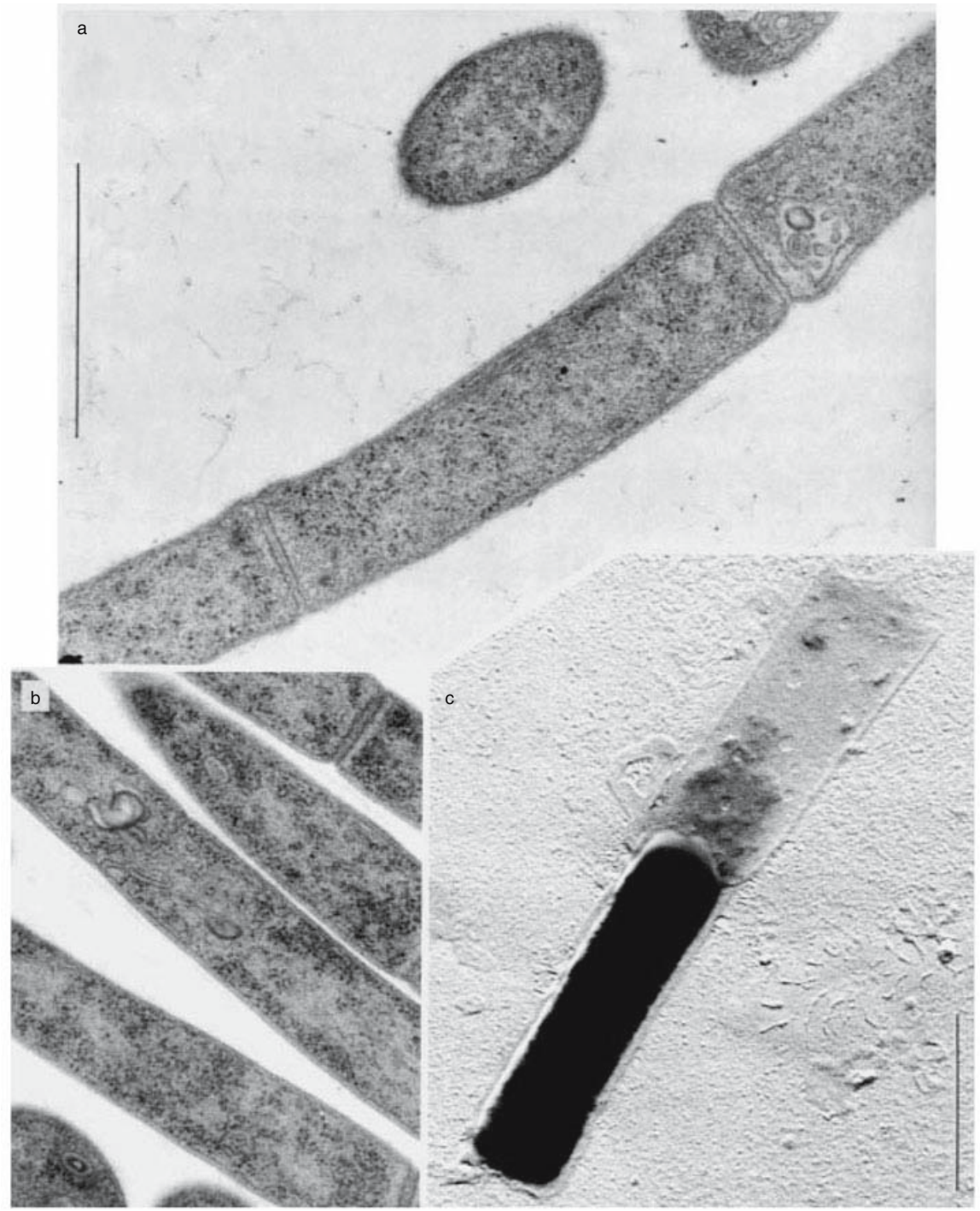

Fig. 5. Electron micrographs of the type strain of *Herpetosiphon aurantiacus*. a) and b) Ultrathin sections; the peptidoglycan layer can be clearly distinguished, but while there is plenty of extracellular slime material, no structure resembling a sheath can be located on the surface; also, the close juxtaposition of the filaments in b) speaks against a sheath. Within the cells, membrane bodies can be seen. c) Shadowed preparation; an end piece with an empty cell-wall cylinder, or sleeve, is visible; again there is no sign of a sheath. Zeiss EM 10B electron microscope. Bars = 1 μm; the magnification is identical for a) and b). (Courtesy of H. J. Hirsch.)

a rough, dry, felt-like surface. Sometimes the swarm surface is completely uniform, with an even, curly, or felt texture. But often elegant networks, massive concentric ridges, a pattern of large and small holes, or large, massive knobs are produced (Fig. 3). These last-mentioned knobs are particularly impressive and have been observed with many different isolates (Holt and Lewin, 1968; Brauss et al., 1969; Reichenbach and Golecki, 1975; Trick and Lingens, 1984; Lee et al. (manuscript in preparation)). Their diameter varies between 200 μm and 2000 μm, and as was noticed repeatedly, they strikingly resemble myxobacterial fruiting bodies, especially since they often are bright red or orange. But the filaments inside those knobs seem not to differ from those in other parts of the swarm, either morphologically or physiologically. Also, the ridges and knobs are not permanent structures but shift continuously from place to place and may even dissolve completely again. This can be seen, e.g., in the movie mentioned earlier (Reichenbach et al., 1980). All those structures seem to originate from chance interactions of the long, migrating filaments that locally pull together, tangle, and pile up. The dynamics of the excreted, drying, and contracting slime may also be involved. Even more spectacular are long, sometimes branched, finger-like protuberances rising up to 5 mm above the swarm surface (Fig. 3). They consist of interwoven filaments and seem to be produced only by certain strains.

Phenotypical and Molecular Characterization

Cell Wall Composition Chemically, the cell wall of *Herpetosiphon aurantiacus* (represented by the type strain ATCC 23779^T and the strain Hp a2 DSM 589) consists of a peptidoglycan that contains L-ornithine in place of diaminopimelic acid, a rather unusual character for a Gram-negative bacterium (Jürgens et al., 1989). A heteropolysaccharide composed of heptose, hexoses, pentoses and *O*-methyl sugars appears to be covalently bound to the peptidoglycan via muramic acid-6-phosphate. This heteropolysaccharide is probably the material seen in electron micrographs as the granular-fibrillar layer on the surface of the longitudinal walls. The peptidoglycan-polysaccharide complex amounts to 20% of the cell dry weight, with a polysaccharide portion of 70%. No evidence for a lipopolysaccharide was found, which would explain the absence of an outer membrane in thin sections; nor was there evidence for the presence of additional sheath material. Unfortunately, the cell wall composition has only been determined for two representants of the *aurantiacus* species group. Nevertheless, the cell wall of *H. aurantiacus* closely resembles that of *Chloroflexus* in both its electron microscopic appearance (Pierson and Castenholz, 1974) and its chemical composition (Jürgens et al., 1987; Meissner et al., 1988). Incidently, the filaments of *Chloroflexus* also often end with a sleeve, which for this organism also has been taken to suggest the existence of a sheath (Pierson and Castenholz, 1974). Hydrolysates of extracellular slime yielded arabinose and glucosamine as the main constituents (for sewage strains; Trick and Lingens, 1984).

Respiratory Quinones The respiratory quinones have only been determined for two strains from the *H. aurantiacus* species group (the type strain *H. aurantiacus* ATCC 23779^T and Hp a2 DSM 589), and for a *Herpetosiphon*-like activated sludge isolate, Wie2, whose genetic relatedness to *Herpetosiphon* (see the sec-tion Taxonomy in this Chapter) is unknown. Mainly menaquinones were detected in these three strains (Kleinig and Reichenbach, 1977; Reichenbach et al., 1978; Senghas and Lingens, 1985). The main menaquinone in *H. aurantiacus* and Hp a2 (DSM 589) is MK-6, and 10% of the respiratory quinone content is MK-7 (Kleinig and Reichenbach, 1977). The respiratory quinones have only been investigated in a few of the culturable representants among chloroflexi. The main common factor seems to be that they contain menaquinones. *Roseiflexus* contains mainly menaquinone-11 (Hanada et al., 2002), *Caldilinea* contains mainly menaquinone-10 under aerobic conditions, whereas no quinones were detected in *Anaerolinea* (Sekiguchi et al., 2003).

Fatty Acid Composition The two type strains of *aurantiacus* and *geysericola* contain the same fatty acids (C16:1, C16:0 and C18:1, cyclopropane C17:0, C17:0 and C18:0), whereas *Herpetosiphon*-like strains show some significant differences to the two type strains (Table 1). Branched-chain fatty acids, which are so typical for many other gliding bacteria, are essentially absent. Hydroxy fatty acids are completely lacking. The novel sulfonolipids, or capnoids, discovered in gliding bacteria of the *Cytophaga* group, were not found in *Herpetosiphon* (Godchaux and Leadbetter, 1983). Only a few fatty acid analyses have been performed on other members of chloroflexi, such as *Chloroflexus*, *Roseiflexus*, *Anaerolinea* and *Caldilinea* (see review in Garrity and Holt [2001] and see Hanada et al. [2002] and Sekiguchi et al. [2003]). However, the results obtained so far clearly indicate that C16:0 is the only common fatty acid present in significant amounts in all these strains and that each strain shows significant differences to the other strains in terms of the composition of other fatty acids.

Table 1. Fatty acid composition in the different species groups of *Herpetosiphon*.

Species	No. of strains	Fatty acid composition
H. aurantiacus[a,b]ATCC 23779[T]	12	C16:1,[a,b] C16:0,[a,b] cyclopropane C17:0,[b] C17:0,[b]C18:1,[a,b] C18:0,[a,b]
H. geysericola[b]ATCC 23076[T]	1	Same composition as the *H. aurantiacus* group
Herpetosiphon-like strains		
Sludge strain Wie2[a]	3	C16:0, C16:1, C18:1, C18:0, C19:0, 8-methyl C17:0
"Soil strains"[b]	10	C14:0, C16:1, C16:0, cyclopropane C17:0, C17:0, C18:1, C18:0, C18:2, C19:1, C19:0, *anteiso* C16

Abbreviation: [T], type strain; C14:0, tetradecanoic acid; C16:0, hexadeconic acid; C17:0, heptadecanoic acid; C18:0, octadecaonic acid; C19:0, nonadecanoic acid.
[a]Senghas and Lingens, 1985.
[b]N. Lee et al., manuscript in preparation.

Pigment Patterns The harvested cell mass and dense colonies on agar plates of *Herpetosiphon* may be brightly colored in shades of yellow-orange to brick-red, but unpigmented strains, especially among *Herpetosiphon*-like strains, do occur (Lee et al. (manuscript in preparation)). However, the pigmentation often depends on the growth medium and is usually more intense on peptone-containing substrates. The pigments are cell bound. Chemically, they are carotenoids (Kleinig and Reichenbach, 1977; Reichenbach et al., 1978). The pigment pattern has so far only been determined for seven strains of the aurantiacus group, including the type strain and two different *Herpetosiphon*-like sludge strains. One of these sewage strains is most likely (unfortunately, these strains are no longer available), on the basis of several phenotypical traits and G+C content, related to the *H. aurantiacus* species group (Trick and Lingens, 1984), whereas the affiliation of the other sewage strain is unknown and is thus placed in an undefined species group (Senghas and Lingens, 1985; see the section Taxonomy in this Chapter). The pigment patterns obtained so far seem however to be uniform since only quantitative (but no qualitative) variation has been observed. The dominant compound has a 4-oxo-beta-psi-chromophore, with a hydroxyl in C-1' to which a disaccharide consisting of glucose is connected via a glycoside bond. One of the sugars bears an ester-bonded fatty acid, in strain Hp a2 (DSM 589) mainly C16:0 and C18:1 (Fig. 6). Composite carotenoids of this type are well known from myxobacteria, but in these latter organisms, only monosaccharides are found as sugar constituents. Carotenoids with a disaccharide component are unique so far among prokaryotic pigments.

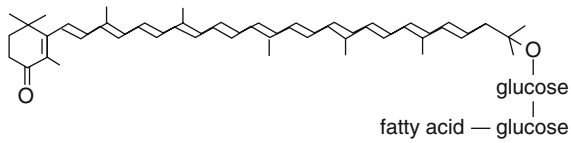

Fig. 6. Chemical structure of the main pigment of *Herpetosiphon aurantiacus*. (From Kleinig and Reichenbach, 1977.)

Metabolic Characteristics Although all known *Herpetosiphon* strains are aerobic, they seem to prefer microaerophilic conditions. All strains, except for the *Herpetosiphon*-like sludge strain of Senghas and Lingens (1985), are catalase- and oxidase positive (Lee et al. (manuscript in preparation)). The nutritional requirements, physiology, and biochemical capabilities of *Herpetosiphon* are not yet fully understood. *Herpetosiphon* is a strict organotroph, but because it is often able to grow on media very low in organic constituents (even on pure water agar, WAT agar; see the section Isolation in this Chapter), the results of substrate-utilization experiments must be interpreted with great care. However, some of the *Herpetosiphon* strains, as well as the *Herpetosiphon*-like sludge strains reported by Senghas and Lingens (1985), do seem to be able to utilize, but not acidify, various hexoses, pentoses and sugar alcohols (Lee et al. (manuscript in preparation)). Organic acids, with the exception of β-hydroxybutyrate, do not support growth.

Apparently, many strains are able to grow on inorganic nitrogen sources (NH_4^+ or NO_3^-), but unequivocal data are often not available. Nitrate reduction to nitrite has only been detected in strains of the *H. aurantiacus* group and some of the *Herpetosiphon*-like sludge organisms (Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)), but only under anaerobic conditions (i.e., in the absence of molecular oxygen), which did not allow growth. Nitrogen fixation has been ruled out in all cases that have been investigated.

Herpetosiphon may contain granules of poly-β-hydroxybutyrate and under certain conditions also of polyphosphate (Trick and Lingens, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)), but analytical details have not been given for a final chemical verification of this observation.

Many *Herpetosiphon* strains show impressive hydrolytic capabilities (e.g., of casein, cellulose, chitin, esculin, gelatin, pectin, starch, tributyrin and xylan; Lee et al. (manuscript in preparation)), while some *Herpetosiphon*-like sludge organisms (Trick et al., 1984; Senghas and Lingens, 1985) and sluice strains (Brauss et al., 1969) are less active in this respect. It should be understood that the results of those digestion experiments sometimes critically depend on the medium and the material used. In the case of pectin, a wide variety of preparations are available that give quite different results. With pectin from apple (38% methyl ester), we found pectin degradation by some of the *Herpetosiphon* strains we tested. Cellulose digestion by different *Herpetosiphon* strains is often seen when using the quick agar test based on CEL1 agar (see below), on which large lysis zones are produced within a few days, reaching far beyond the swarm colony. This suggests that cellulose decomposition by *Herpetosiphon* is achieved by diffusible exoenzymes (Lee et al. (manuscript in preparation)). However, only *H. geysericola* (ATCC 23076^T), Hp a1 (DSM number in preparation) and strain So97 (DSM 14854) decompose filter paper, but decomposition is usually slow (recognizable only after 20–30 days at 30°C on ST6 Agar; Lewin, 1970; Lee et al. (manuscript in preparation)).

CEL1 Agar

Cellulose powder	0.5%
$(NH_4)_2SO_4$	0.1%
Agar	1%

Dissolve ingredients in distilled water, adjust to pH 7.2. After autoclaving, pour the medium as a thin layer on top of ST6 agar plates. A suitable cellulose powder, MN 300, is produced by Macherey and Nagel (Germany).

ST6 Agar

Part A

$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$MnSO_4 \cdot 2H_2O$	0.01%
$FeCl_3 \cdot 6H_2O$	0.02%
Trace element solution (same as for medium MD1)	1 ml/liter

Ingredients of part A should be dissolved in one-third of the water (*distilled*) volume. Ingredients of part B should be dissolved in two-thirds of the water volume.

Part B

K_2HPO_4	0.1%
Yeast extract	0.002%
Agar	1%

After autoclaving parts A and B separately, combine them and pour the mixture into plates.

With many strains, we have also observed DNA hydrolysis on DNA agar, but in some studies of *Herpetosiphon*-like sludge organisms, tests for DNase were negative (Senghas and Lingens, 1985). Not all strains show β-hemolysis of sheep and human erythrocytes (Gräf and Perschmann, 1970; Trick and Lingens, 1984; Lee et al. (manuscript in preparation)).

Most investigators report that *Herpetosiphon* organisms lyse dead bacteria but also in a few cases live bacteria. However, not all types of bacteria are equally sensitive. For instance, Quinn and Skermann (1980) have reported that some *E. coli*, as well as some Gram-positive strains (*Bacillus subtilis*, *Lactobacillus casei* and *Streptococcus mutans*), turned out to be particularly recalcitrant (Lee et al. (manuscript in preparation)). Not all *Herpetosiphon* strains show identical lysis patterns. The *Herpetosiphon*-like sludge organisms of Trick and Lingens (1984) and Senghas and Lingens (1985) do not lyse bacteria at all; however, the *Herpetosiphon aurantiacus* type sludge organism FC16a (DSM number in preparation) of N. Lee et al. (manuscript in preparation) does lyse bacteria. The autoclaved yeast cells in VY/2 agar are often but not always destroyed. Other yeast species may also be lysed, such as *Pichia stipitis* or *Candida albicans* (Lee et al. (manuscript in preparation)).

The H_2S, indole and acetoin tests were negative for all strains tested, whereas the phosphatase and the urease tests were positive for some of the strains tested (Trick and Lingens, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). Tyrosine in tyrosine agar is often degraded.

Tolerance Tests

The *Herpetosiphon*-like sludge organisms tolerate up to 2% NaCl, but most other *Herpetosiphon* strains are rather sensitive to elevated salt concentrations; growth is rarely observed above 1.5% NaCl. Optimum pH for all strains is around 7; however, growth is tolerable in the pH range 4–10. Optimum temperature for all *Herpetosiphon* strains is 20–30°C, but growth is generally seen in the range 10–37°C. *Herpetosiphon*-like strains (e.g., Hp g207, DSM 15737) may grow at 45°C (Trick and Lingens, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). All *Herpetosiphon* strains investigated so far are resistant to high levels of kanamycin sulfate (250 mg/liter).

Molecular Characterization

The G+C content of the two *Herpetosiphon* species groups (*aurantiacus* and *geysericola*) is 48–53 mol%. However, as already mentioned in the introduction, the G+C content of some *Herpetosiphon*-like isolates, e.g., the strain of Senghas and Lingens (1985), and the strains Hp g6 DSM

15710, Hp g16 DSM 15736 and Hp g207 DSM 15737 is higher (59–75%), which suggests that at least some of these isolates may represent new genera among chloroflexi (Lee et al. (manuscript in preparation)).

The unique multicopy single-stranded DNA (msDNA) common in gliding myxobacteria and also found in *Flexibacter* could not be demonstrated in *Herpetosiphon* (Dhundale et al., 1985). The base sequence of the 5S rRNA gene has only been determined for one of the *Herpetosiphon*-like sludge organisms (strain Wie 2; Van den Eynde et al., 1987). While this 5S rRNA gene sequence corresponds, in general, quite well with the model of eubacterial 5S rRNA structure, there are several deviations—in two cases in highly conserved sites not modified in any other eubacterium. The base sequence of the 16S rRNA gene has been determined for 15 different *Herpetosiphon* strains (Oyaizu et al., 1987; Bradford et al., 1996; Sly et al., 1998; Kohno et al., 2002; Lee et al. (manuscript in preparation)). Comparative 16S rRNA gene sequence analyses confirmed that they all form a specific *Herpetosiphon* cluster within chloroflexi (see the section Taxonomy in this Chapter).

Table 2. 16S rRNA gene sequence similarity values of *Herpetosiphon* strains and other culturable chloroflexi strains.

Culturable chloroflexi strains	Similarity to 16S rRNA gene[a] (%)
H. aurantiacus strains (14 strains[b])	97.8–99.5
H. geysericola	96.4
Herpetosiphon-like strains (7 strains[c])	79–94
Chloroflexus aurantiacus	81.6
Chloroflexus aggregans	80.3
Chloronema giganteum	81.8
Kouleothrix aurantiacus	82.8
Roseiflexus castenholzii	82.6
Oscillochloris trichoides	82.8
Eikelboom type 1851 Ben52	81.3

[a]The gene of *H. aurantiacus* ATCC 23779T.
[b]Hp g8 (DSM 6205), Hp g10 (DSM 6207), HrsBendi 15 FO18 (Bradford et al., 1996), OSI-B2 (Kohno et al., 2002), FC16a (DSM number in preparation), Hp al (DSM number in preparation), Hp a2 (DSM 589), Hp g5 (DSM number in preparation), Hp g156 (DSM 15901), Hp g158 (DSM 15902), Hp g254 (DSM 15903), Hp g269 (DSM 15712), Hp g277 (DSM 15713), and Hp g278 (15714).
[c]Hp g6 (DSM 15710), Hp g16 (DSM 15736), Hp g207 (DSM 15737), Hp g122 (DSM 15874), Hp g124 (DSM 15875), Hp g150 (DSM 15876), and Hp g174 (DSM 1587).
The data for *H. geysericola* are from Sly et al., 1998, whereas the rest of the data are preliminary and from N. Lee et al. (manuscript in preparation).

Taxonomy

The closest culturable relatives of *Herpetosiphon* are only distantly related to *Herpetosiphon* (e.g., *Chloroflexus aurantiacus* and *Roseiflexus aurantiacus*, showing around 81–83% 16S rRNA gene similarity; Table 2). This has been deduced from different comparative 16S rRNA gene sequence studies, from analyses of oligonucleotide catalogs via binary association coefficients (S_{AB} values), through early oligonucleotide signatures (Gibson et al., 1985; Oyaizu et al., 1987) and from a recent comparison of the nearly full 16S rRNA gene sequence of around 15 different strains of *Herpetosiphon* (Lee et al. (manuscript in preparation)), as well as from comparative sequence analysis of elongation factor Tu and ATP–synthase beta-subunit genes (Ludwig et al., 1993). Together with *Anaerolinea* gen. nov., *Caldilinea* gen. nov., *Chloronema*, *Heliothrix*, *Roseiflexus*, *Oscillochloris*, "the Eikelboom type 1851 sludge strain" (Beer et al., 2002), and the *Nostocoida limicola*-like strains of Schade et al. (2002), they represent the few culturable organisms in chloroflexi (<10% of all submitted chloroflexi-affiliated sequences in general molecular databases, June, 2004). Interestingly, most of these culturable strains consist mainly of thermophilic phototrophs (*Chloroflexus*, *Chloronema*, *Heliothrix*, *Roseiflexus* and *Oscillochloris*) or thermophilic organotrophs (*Anaerolinea* gen. nov. and *Caldilinea* gen. nov.). The only exceptions are *Herpetosiphon*, the sludge strains Eikelboom type 1851, the *Nostocoida limicola*-like strains of Schade et al. (2002), and a few strains in different thermophilic genera that are mesophilic organotrophs. At the present stage, the *Herpetosiphon* strains represent the largest group of culturable, nonthermophilic, nonphotosynthetic organisms among the chloroflexi.

Since the discovery of the first *Herpetosiphon* strain in 1968 by Holt and Lewin, the genus *Herpetosiphon* is today still only represented by two rather similar species, *H. aurantiacus* and *H. geysericola,* and some *Herpetosiphon*-like sludge strains (Trick and Lingens, 1984; Senghas and Lingens, 1985; Bradford et al., 1996), whose genetic relatedness to *Herpetosiphon* can no more be determined since the strains are no longer existent. Thus, *Herpetosiphon* is the only genus in the family Herpetosiphonaceae in the second order of the class *Chloroflexi*, *Herpetosiphonales*. As mentioned earlier (see the section Habitats in this Chapter), the three marine "*Herpetosiphon*" strains, which were discovered by Lewin (1970), have recently been transferred to another phylum, *Flexibacter-Bacteroides-Cytophaga* (Sly et al., 1998). Since the discovery of the second culturable *representative of the Class Chloroflexi, Chloroflexus aurantiacus* (Pierson et al., 1974), a vast amount of as yet not cultivatable strains of *chloroflexi*-affiliated organisms have been detected (see, e.g., the review of Björnsson et al., 2002). Only a few of these studies have reported any *Herpetosiphon*-

Table 3. Fatty acid composition, G+C content, 16S rRNA gene similarity and DNA-DNA similarity in different *Herpetosiphon* and *Herpetosiphon*-like strains.

Species groups of *Herpetosiphon*	No. of strains	FA[a]	G+C mol%	16S rRNA similarity[b] (%)	DNA-DNA similarity[b] (%)
H. aurantiacus[c] ATCC 230779^T	12	I	48.1–52.9	97.8–99.5	65.3–95
H. geysericola[c,d] ATCC 23076^T	1	I	51.8	96.4	67.8
Herpetosiphon-like strains:					
"Sludge strains"[e]	3	II	59	ND	ND
"Sludge strains"[f]	5	ND	48.7–49.0	ND	ND
"Soil strains"[c]	10	III	62–75	81–95	<15

Abbreviations: FA, fatty acid composition category; ^{T}, type strain; and ND, not determined, strains no longer available.
[a]Each group (I to III) represent a unique composition; see Table 1.
[b]To the type strain *H. aurantiacus* (ATCC 23779^T).
[c]N. Lee et al. (manuscript in preparation).
[d]Sly et al. (1998).
[e]Senghas and Lingens (1985).
[f]Trick and Lingens (1984).

like organisms, which suggests that the *Herpetosiphon* group might represent a rather stagnant evolutionary group of organisms with a low diversity among the *chloroflexi*. However, recent studies by Lee et al. (manuscript in preparation)) showed that the genetic diversity among the *Herpetosiphon*-like strains is much larger than previously expected, differing up to 19% in 16S rRNA gene sequence from the type strain of *H. aurantiacus*, which suggests that some of these strains represent novel genera among chloroflexi. Thus, the taxonomy of the phylum *Chloroflexi* and the order *Herpetosiphonales* may have to be re-evaluated in the future. Table 3 gives the distinguishing characteristics of the two recognized *Herpetosiphon* species and the *Herpetosiphon*-like organisms.

Summary of the Species Groups of *Herpetosiphon*

H. AURANTICUS. This species group, represented by the type strain ATCC 23779^T and isolated by Lewin and Holt (1968), contains the largest number of strains (14, listed below). The G+C content is 48.1–52.9 mol%. 16S rRNA gene similarity to the type strain is between 97.8% and 99.5% (Table 2). However, the DNA-DNA similarity to the type strain may vary from 65.3% to 95% (Table 3). The fatty acid composition is similar in all strains (Table 1), but the strains may differ in some single phenotypical characteristics (Lee et al. (manuscript in preparation)). Strains have been isolated from freshwater lakes (Lake Constance, Germany: Hp g8 [DSM 6205], Hp g10 [DSM 6207]; Gräf et al., 1970; Lee et al. (manuscript in preparation)); from activated sludge in different parts of the world: HrsBendi 15 FO18 (Bradford et al., 1996), OSI-B2 (Kohno et al., 2002), FC16a [DSM 15711] (Lee et al. (manuscript in preparation)); and from soil from different parts of the world: Hp a1 (DSM number in preparation), Hp a2 (DSM 589), Hp g5 (DSM number in preparation), Hp g156 (DSM 15901), Hp g158 (DSM 15902), Hp g254 (DSM 15903), Hp g269 (DSM 15712), Hp g277 (DSM 15713), Hp g 278 (DSM 15714) by H. Reichenbach and by Lee et al. (manuscript in preparation).

H. GEYSERICOLA. This species is represented by only one strain, the type strain *H. geysericola*, ATCC 23076^T (described by Lewin, 1970). Originally it was suggested (Lewin, 1970) that *H. geysericola* is identical with *Phormidium geysericola* (Copeland, 1936), but this appears very unlikely, because *Phormidium geysericola* was found at the rim of a geyser and in 60–84°C water from various alkaline hot springs in Yellowstone. It thus is definitely a thermophile and would hardly grow at 30°C, as does *H. geysericola*. The validity of *H. geysericola*´s species status has been discussed, since it shares several common phenotypical characteristics (exceptions: urease reaction, nitrate reduction, and hydrolysis of cellulose filter paper), G+C content, and fatty acid composition with the *H. aurantiacus* group (Reichenbach, 1992; Lee et al. (manuscript in preparation)). Recent studies have shown that the 16S rRNA similarity (96.4%; Sly et al., 1998; Table 2) and the DNA-DNA similarity (67.8%, Lee et al. (manuscript in preparation); Table 3) to *H. aurantiacus* is on the border of the present species definition (Stackebrandt et al., 2002). Since the DNA similarity between some of *H. aurantiacus* strains may also range from 65.3% to 95% (Table 3), it is questionable whether *H. geysericola* should remain a distinct species. The main difference between *H. geysericola* and all 14 *H. aurantiacus* strains is that *H. geysericola* was isolated from the vicinity of a hot spring and thus was defined as "thermophilic," although the proper characterization should be thermotolerant. However, thermotol-

erance is not unusual with *aurantiacus* strains, which may also grow at 38–40°C Lee et al. (manuscript in preparation). Further taxonomical studies on *H. geysericola* (in particular investigation of chemotaxonomical characteristics) and, if possible, on more *geysericola*-like strains retrieved from sites in and around hot springs will be needed to determine whether *H. geysericola* should remain a separate species or be included in the *H. aurantiacus* group. Another theoretical possibility is also that some of the present strains within the *H. aurantiacus* group should be transferred to *geysericola*, although they were not isolated from sites in and around hot springs but from soil. The reason for this is that their DNA-DNA similarity values to the *H. aurantiacus* type strain approximates that of the present species definition (i.e., slightly below 70% similarity). Further DNA-DNA similarity studies with these strains would be needed to clarify this question.

Herpetosiphon-LIKE STRAINS. In the first attempt after isolation, several strains have been identified as *Herpetosiphon*-like, but further studies, in particular, utilizing molecular techniques (16S rRNA gene sequence analysis), have revealed that the diversity of *Herpetosiphon*-like strains is much larger than expected. The 16S rRNA gene similarity between *Herpetosiphon*-like strains and *H. aurantiacus* (ATCC 23779^T) is around 79–94%, and the G+C content is around 62–75% (Lee et al. (manuscript in preparation); Table 3). Thus, these strains represent most likely new genera among the chloroflexi, e.g., the strains Hp g6 (DSM 15710), Hp g16 (DSM 15736), Hp g207 (DSM 15737), Hp g122 (DSM 15874), Hp g124 (DSM 15875), Hp g150 (DSM 15876), and Hp g174 (DSM 1587). All these strains have been isolated from soil Lee et al. (manuscript in preparation). The 16S rRNA genes of the *Herpetosiphon*-like sludge organisms described by Trick and Lingens (1984) and Senghas and Lingens (1985) unfortunately cannot be sequenced, since these strains are no longer available. However, previous studies, based on analysis of oligonucleotide catalogs via binary association coefficients (S_{AB} values), showed that the *Herpetosiphon*-like sludge organisms isolated by Senghas and Lingens (1985) are clearly related but not identical to *Herpetosiphon* (S_{AB} values with *Herpetosiphon*: 0.39, for *Chloroflexus*: 0.40, and *Herpetosiphon-Chloroflexus*: 0.31). This is also indicated by the significantly higher G+C mol% value obtained for these organisms (Table 3).

Microbial Ecology

We know virtually nothing about the true distribution, dynamics and activity of *Herpetosiphon* in the environment, as well as about its interactions with other organisms, such as other bacteria or eukaryotic grazers. No reports on bacteriophages of *Herpetosiphon* have been published. All we know is that *Herpetosiphon* may be found in freshwater and sewage systems, in the vicinity of hot springs, as well as in diverse types of soil systems in different countries and islands in many parts of the world (Africa, America, Australia, Europe, India, Japan and Mexico). Our knowledge about the biogeography of *Herpetosiphon* is mainly based on isolation studies or surveys using different chloroflexi specific probes detected by means of FISH (Björnsson et al., 2002; Lee et al. (manuscript in preparation)). Only a few 16S rRNA clone library or denaturing gradient gel electrophoresis screening studies on the microbial community composition in freshwater lakes or sewage systems have reported *Herpetosiphon*-like 16S rRNA genes (Gich et al., 2001; Kohno et al., 2002), despite the fact that a constantly increasing amount of novel, in most cases, chloroflexi-like sequences (from as yet noncultivable organisms, not related to *Herpetosiphon*) are being found in nearly all kinds of environments all over the world (e.g., Pierson et al., 1985; Giovannoni et al., 1996; Snaidr et al., 1997; Chandler et al., 1998; Dojka et al., 1998; Hugentholtz et al., 1998a; Hugentholtz et al., 1998b; Gordon et al., 2000; Sekiguchi et al., 2001; Alfreider et al., 2002; Bano and Hollibaugh, 2002; Boomer et al., 2002; Dunbar et al., 2002; Furlong et al., 2002; Hentschel et al., 2002; Juretschko et al., 2002; Lee et al. (manuscript in preparation)). However, as pointed out earlier (in the section Habitats in this Chapter), this does still not provide the final evidence for the nonexistence of *Herpetosiphon* in any of these systems. It may be that search for *Herpetosiphon* is negatively biased in general molecular screening studies (Lee et al. (manuscript in preparation)), so that specific molecular markers for *Herpetosiphon* must be used to detect them. In those cases, where a large attempt has been undertaken to isolate bacteria from the environment, none of the media used were optimal for the isolation of *Herpetosiphon*.

Whereas virtually nothing is known about the abundance of *Herpetosiphon* in soil, some observations, albeit contradictory, on the abundance of *Herpetosiphon* and other filamentous chloroflexi have been made in sewage plants using different chloroflexi-specific oligonucleotides in FISH (Fig. 7). Björnsson et al. (2002) reported that the amount of chloroflexi filaments in subphylum III (to which *Herpetosiphon* is affiliated) was considerably higher than the amount of other chloroflexi filaments affiliated to other subphyla. N. Lee et al. (manuscript in preparation) found the contrary. Unfortunately, nothing is

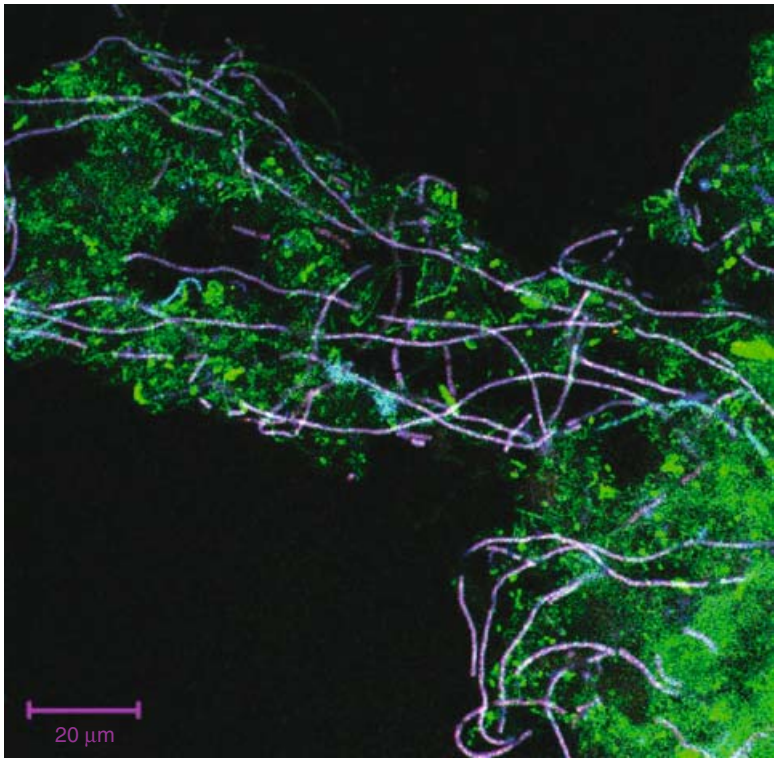

Fig. 7. Image of fluorescence *in situ* hybridization using oligonucleotide probes targeting chloroflexi filaments on the phylum level (probe CFX1223 in Cy3, red) and on the subphylum III level (to which *Herpetosiphon* belongs, CFX784 probe in Cy5, blue) and Bacteria (EUB338, in FLUOS-Prime, green) in activated sludge (photo: N. Lee; for probe references, see Björnsson et al., 2002). The purple-colored filaments are a result of overlapping Cy3 (red) and Cy5 (blue) stained cells. Bar = 20 µm.

known about the *in situ* abundance of *Herpetosiphon* in other sewage systems where sequences of other culturable and as yet not cultivable chloroflexi strains have been obtained (e.g., Bradford et al., 1996; Beer et al., 2002; Juretschko et al., 2002; Kohno et al., 2002; Onuki et al., 2002; Schade et al., 2002). In those cases where isolation attempts were undertaken, no media were used that were optimal for *Herpetosiphon*, and in those cases where FISH was used, no probes targeting specifically *Herpetosiphon* were applied, and so no definitive conclusions about their nonexistence in these systems can be drawn. Thus, the contradiction between the observations made by Björnsson et al. (2002) and Lee et al. (manuscript in preparation) is most likely explained by the effect of unknown geographical factors or operational differences between the different sewage systems. As long as nothing is known about the role of *Herpetosiphon* in sewage systems and about whether significant physiological differences exist among these sewage *Herpetosiphon* strains, any attempt to explain the differences in abundance of *Herpetosiphon* will remain speculative. Considering the hydrolytic capabilities of *Herpetosiphon*, it may however be assumed that the bacterium participates in the degradation of macromolecules and perhaps in the control of microbial populations in nature (Quinn and Skerman, 1980; Lee et al. (manuscript in preparation)).

Molecular Genetics

Nothing is known about the genetics of *Herpetosiphon*. Also, whether *Herpetosiphon* possesses mobile genetic elements or is able to acquire plasmids through lateral gene transfer is unknown. The genome of *Herpetosiphon* has not been sequenced, but the genome of its closest culturable relative, the photosynthetic *Chloroflexus*, is currently being sequenced in a vast program aimed at investigating the evolution and genetics of photosynthesizing prokaryotes (DOE Joint Genome Institute, United States Department of Energy). Future sequencing of the genome of *Herpetosiphon* might however yield interesting insights into the evolution of early life on earth and the divergence of the different members among the present representatives in chloroflexi. A possible case for lateral gene transfer in *Chloroflexus* has already been indicated by a comparative phylogenetic analysis of circadian clock genes in *Archaea* and *Bacteria* showing that these genes might have been laterally transferred from cyanobacteria to different taxa (among them *Chloroflexus*) in *Archaea* and *Bacteria* (Dvornyk et al., 2003).

Given the unique position of the phylum *Chloroflexi*, it is not unlikely that further studies on the molecular genetics and biochemistry of *Herpetosiphon* may reveal interesting facts. This has been shown to be the case in screening studies of

two different enzymes in some *Herpetosiphon* strains from the *H. aurantiacus* species group: the endoribonucleases (Haas and Brown, 1998) and the restriction endonucleases (Mayer and Reichenbach, 1978). The ribonuclease P has been sequenced for several classical phyla of *Bacteria* (encompassing 145 different strains, including three chloroflexi strains, *Chloroflexus*, *Herpetosiphon* and *Thermomicrobium*) for further evolutionary comparisons and revealed interesting insights into the structural evolution of the RNA molecule (Haas and Brown, 1998). A whole family of 17 restriction endonucleases has been more or less completely characterized with respect to their recognition and cleavage specificities (Brown et al., 1980; Kröger et al., 1984; Düsterhöft et al., 1991). All appear to be class II endonucleases, producing cohesive ends, either in the 5′-strand or, in the cases of endonucleases *Hgi* AI and *Hgi* JII, in the 3′-strands, and seem to be located in the periplasm. Most of them interact with hexanucleotide sequences and some with pentanucleotide sequences, but virtually all recognition sequences are degenerate. The availability of such a large family of endonucleases is of considerable theoretical interest because it allows a comparative study of the mechanisms of protein-DNA interaction as well as of molecular evolution. The known *Herpetosiphon* enzymes can be arranged in such a way that their recognition sequences overlap and the whole set thus becomes a continuous system (Kröger et al., 1984). One of the enzymes, *Hgi* EII, has a unique recognition sequence of 2×3 bases separated by 6 unspecified nucleotides. When the recognized bases are read in sequence, they give the recognition sequence of *Hgi* CI, and it has been proposed that enzyme *Hgi* EII originated by an inversion of the two enzyme subunits so that the recognition sites are now on the surface rather than in the center of the enzyme. Isoschizomeres can be found for almost all *Herpetosiphon* recognition sequences, e.g., *Sal* I enzyme is isoschizomere to *Hgi* CIII and *Hgi* DII. Interestingly, no hybridization was found between *Sal* I gene probes and *Herpetosiphon* DNA, whereas two other producers of *Sal* I isoschizomeres, *Rhodococcus rhodochrous* and *Xanthomonas amaranthicola*, showed clear DNA similarity under stringent conditions (Rodicio and Chater, 1988). While this suggests that the latter organisms and *Streptomyces albus* G have at least partially homologous enzymes, the high G+C difference of 20 mol% between *Herpetosiphon* and *Streptomyces* could have obscured, through codon replacement, a structural relationship between their enzymes. A comparison of the base sequences of the genes or of the amino acid sequences of the various enzymes should answer the question.

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