Description and characterization of bacteria attached to lotic organic aggregates (river snow) in the Elbe River of Germany and the South Saskatchewan River of Canada

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Abstract

Aerobic and anaerobic cultivation techniques, 16S rDNA based phylogeny, and fluorescent in situ hybridization (FISH) were used to describe the phylogenetic diversity and physiological versatility of lotic microbial aggregates (river snow) obtained from the river Elbe. In the course of the year, the river snow community was characterized by a great bacterial diversity in spring with total bacterial cell counts of 2.5 x 10^8 cells per ml, the predominant occurrence of algae in summer (total bacterial cell counts 2.0 x 10^8), and the reduction of total bacterial cell counts in autumn 1.2 x 10^8 and winter 1.4 x 10^8 (all mean values). In all river snow samples, more than 70% of the bacteria, counted with the general DNA stain DAPI, also hybridized with the *Bacteria* specific probe EUB338. In situ analysis of the bacterial river snow community with a comprehensive suite of specific rRNA targeted probes revealed population dynamics to be governed by seasonal factors. During all seasons, beta-*Proteobacteria* constituted the numerically most important bacterial group forming up to 54% of the total cell counts. In contrast to this, the relative abundance of other major bacterial lineages ranged from 2% for the order *Planctomycetales* to 36% for *Cytophaga-Flavobacteria*. Batch cultures of river snow samples fed with sterile Elbe river water (0.2-µm-pore-size filtered) and supplemented with minimal amounts (0.1% w/v) of different substrates resulted in remarkable changes of the microbial community composition, with N-acetylglucosamine favouring the growth of beta-*Proteobacteria.* Cultivation of river snow under aerobic and anaerobic conditions with a variety of different media resulted in the isolation of 40 bacterial strains. Phenotypical and phylogenetical analysis revealed them as mostly unknown organisms affiliated to different bacterial phyla. Application of newly developed specific oligonucleotide probes proved the cultivated bacteria, including *Aeromonadaceae,* affiliated to the gamma-*Proteobacteria*, clostridia and the

numerically abundant beta-*Proteobacteria,* as in situ relevant members of the river snow community.

For the simultaneous detection of cellular components and extracellular polymeric substances (EPS) in lotic microbial aggregates (river snow) a new technique combining fluorescent in situ hybridization and lectin-binding-analysis (FISH-LBA) was developed. River snow aggregates were directly collected from the bulk water phase into coverslip chambers, in which the complete procedure including fixation, fluorescent in situ hybridization, lectin-binding and optical analysis by confocal laser scanning microscopy was performed. Neither autofluorescence originating from photosynthetic organisms nor inorganic particles did negatively interfere with the FISH-LBA technique. In Elbe river snow samples distinct compartments of the river snow structure could be visualized with FITC-labelled lectins from *Triticum vulgaris*, *Limulus polyphemus*, *Arachis hypogaea, Phaseolus vulgaris* and *Pseudomonas aeruginosa*, each binding to frequently occurring different saccharide residues in the EPS matrix. The analysis could be performed on different levels of complexity. The new combined technique visualized bacteria of different phylogenetic groups in the entire river snow structure as well as EPS components linked with various microcolonies. Slime-layers and cell-envelopes of individual eucaryotic and procaryotic cells could also be observed.

Cultivation and isolation of members of the bacterial river snow community of the Canadian South Saskatchewan River on the oligotrophic medium FBM led to the discovery of the bacterial strain F8. 16S rDNA sequencing and phylogenetic analysis revealed this isolate as a deep branching gamma-*Proteobacterium*. Strain F8 was generally noticed by a remarkable kind of a self-produced filamentous network. The process started with rod shaped bacterial cells, accumulating a self-produced material around them, followed by the formation of filaments of different length. Filament formation developed into a network in form of a sponge like pattern. Cells

moved along this network assembling firstly small and subsequently larger clusters. The process ended with large aggregates of cells, leaving an empty network of filaments behind which could be observed in cultures growing on solid and/or liquid media. Although strain F8 was able to grow in a variety of different media, filament formation occurred only in low nutrient media. Filaments could be stained with different dyes. The presence of filaments could be revealed by light microscopy, confocal laser scanning microscopy (CLSM) as well as by transmission electron microscopy (TEM).

Zusammenfassung

In den Schwebstoffflocken der Fliessgewässer spielen Bakterien neben Algen, Pilzen, Protozoen, anorganischen Partikeln und organischem Detritus eine wesentliche Rolle. Am Beispiel der Elbe wurde die phylogenetische Diversität und Physiologie dieser aquatischen Bakterien mittels molekularbiologischer Methoden (in situ Hybridisierung, 16S rDNS Sequenzanalyse), aerober und anaerober Kultivierung, sowie konfokaler laser scanning Mikroskopie untersucht. Im Jahresverlauf zeigte sich, dass die mikrobielle Lebensgemeinschaft der Schwebstoffflocken in Frühjahrsproben durch eine grosse bakterielle Diversität gekennzeichnet war. In Sommerproben dagegen dominierten unterschiedliche Cyanobakterien und Grünalgen. Herbst- und Winterproben wiesen eine deutliche Abnahme der bakteriellen Gesamtzellzahl auf. Mehr als 70% aller mit DAPI nachgewiesenen Bakterien konnten mit der Eubakterien spezifischen Sonde EUB338 detektiert werden. Die in situ Analyse der Schwebstoffflocken mit unterschiedlichen Oligonukleotid Sonden, zeigte, dass die überwiegende Mehrheit der phylogenetischen Bakteriengruppen jahreszeitlichen Schwankungen mit Werten von 2% für die Planktomyceten bis zu 36% für die Cytophagen-Flavobakterien Gruppe unterlagen. Im Gegensatz dazu, waren die beta-*Proteobakterien* in allen Jahreszeiten anzutreffen, und erreichten mit Werten zwischen 50-54% von der Gesamtzellzahl die höchsten Zellzahlen überhaupt. Statische Schwebstoffflockenkulturen, angesetzt mit steril filtriertem Elbewasser und angereichert mit Spuren verschiedener Substrate, führten zu morphologischen und phylogenetischen Veränderungen der mikrobiellen Lebensgemeinschaft. Zugabe von N-acetylglucosamin ergab einen deutlichen Anstieg der beta-*Proteobakterien*. Die aerobe und anaerobe Kultivierung auf einer Vielzahl verschiedener Medien führte zur Isolierung von 40 Bakterienreinkulturen aus unterschiedlichen phylogenetischen Gruppen. Mit Hilfe neu entwickelter, spezifischer Oligonukleotidsonden, konnten die

isolierten Bakterien als relevante und dominante Vertreter der mikrobiellen Lebensgemeinschaft ermittelt werden.

Für die gleichzeitige Erfassung zellulärer Komponenten und extrazellulärer polymerer Sustanzen (EPS) wurde im Rahmen dieser Arbeit eine neue kombinierte Technik aus Fluoreszenz in situ Hybridisierung und Lektin-Bindungs-Analyse (FISH-LBA) entwickelt. Die Schwebstoffflocken wurden direkt aus dem Elbewasser in Objektträgerkammern überführt, in denen die komplette Präparation, bestehend aus Fixierung, FISH, LBA und anschließender mikroskopischer Analyse, durchgeführt wurde. Anorganische Partikel aus den Schwebstoffflocken, wie auch die Autofluoreszenz von photosynthetischen Organismen, hatten keinen störenden Einfluß auf die neue Methode. FITC markierte Lektine von *Triticum vulgaris*, *Limulus polyphemus*, *Arachis hypogaea, Phaseolus vulgaris* und *Pseudomonas aeruginosa* konnten durch Bindung an die jeweils spezifischen Zucker in der EPS-Matrix der Schwebstoffflocken bestimmte Bereiche innerhalb der Flocken sichtbar machen. Die neue Methode ermöglichte die Detektion von einzelnen Bakterien und Mikrokolonien unterschiedlicher phylogenetischer Gruppen mit den sie umgebenden EPS Komponenten in verschiedenen Bereichen der Flocken. Darüber hinaus war auch die chemische Identifizierung von Bestandteilen der Schleimkapseln und Zellhüllen einzelner eukariontischer und prokariontischer Zellen möglich.

Kultivierung der bakteriellen Lebensgemeinschaft der Schwebstoffflocken aus dem South Saskatchewan River in Saskatchewan, Kanada, auf verschiedenen Medien führte zur Isolierung der bakteriellen Reinkultur F8. Durch 16S rDNS Sequenzierung und phylogenetische Analyse konnte die Zugehörigkeit des Isolates F8 zur Gruppe der gamma-*Proteobakterien* ermittelt werden. Stamm F8 fiel bei mikroskopischen Beobachtungen durch eine bemerkenswerte Interaktion der einzelnen bakteriellen Zellen entlang eines selbst produzierten filamentösen Netzwerkes auf. Der koordinierte Bewegungsablauf startete mit der Akkumulierung eines von den Zellen

produzierten Materials, was sich im weiteren Verlauf zu Filamenten unterschiedlicher Länge umformte. Diese Filamente bildeten daraufhin ein komplexes Netzwerk, an dem sich die Zellen entlang bewegten. Die Zellen schlossen sich zunächst in kleineren und später in größeren Gruppen zusammen. Der Prozess endete mit grossen Zellanhäufungen und einem zellfreien Netzwerk aus Filamenten. Dieser Prozess konnte sowohl bei Kultivierung auf Fest- als auch in Flüssigmedien beobachtet werden. Obwohl der Stamm F8 in der Lage war, auf einer Vielzahl unterschiedlicher Medien zu wachsen, trat die Bildung von Filamenten nur in nährstoffarmen Medien auf. Die Filamente konnten mit verschiedenen Farbstoffen angefärbt werden. Ihre Anwesenheit konnte sowohl lichtmikroskopisch als auch durch konfokale laser scanning Mikroskopie und Elektronenmikroskopie nachgewiesen werden.

Abbreviations

A	adenine
AHL	N-acetyl homoserine lactones
AMM	anaerobic mineral medium
API-E	identification system for *Enterobacteriaceae*
API-NE	identification system for non-enteric Gram-negative rods
ARDRA	amplified ribosomal DNA restriction analysis
C	cytosine
CD	Czapek-Dox medium
CF	Cytophaga and Flavobacteria medium
CLSM	confocal laser scanning microscopy
Cy3	indocarbocyanine
Cy5	indodicarbocyanine
DAPI	4`,6-diamidino-2-phenylindole
DNA	desoxynucleic acid
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polymeric substances
FA	formamide
FBM	freshwater basal medium
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
G	guanine
glcAc	D-glucuronic acid
GN	Gram-negative
M	base A or T
NB	nutrient broth
NeuNAc	N-acetylneuraminic acid

PBS	phosphate buffered saline
PCR	polymerase chain reaction
R	base A or G
RCM	reinforced clostridial agar
rDNA	ribosomal desoxynucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RRW	raw river water
SDS	sodium dodecyl sulfate
SRB	sulfate-reducing bacteria
SSU	small subunit
T	thymine
TE	Tris EDTA
TEM	transmission electron microscopy
Tris	tris-(hydroxymethyl)-aminomethane
TRITC	tetramethyl rhodamine isothiocyanate
TSA	tryptic soy agar
Tween40	polyoxyethylenesorbitane monopalmitate
Tween80	polyoxyethylenesorbitane monooleate
v/v	volume/volume
W	base A or T
w/v	weight/volume
Y	base C or T

Table of contents **Page**

1. Introduction

In the global hydrological cycle, lotic systems are the major linkage between terrestrial and aquatic habitats, including surface, groundwater and the oceans. The characteristic feature of the river ecosystems is the continuous movement of the running water. Streams and rivers are temporary locations for the storage and transport of a broad range of different materials. Running waters are enormously diverse with respect to their dimensions, ranging from small creeks to great streams, and they occur under widely different conditions in terms of climate, vegetation, topography and geology. However, physical and chemical parameters are not sufficient to get a comprehensive understanding of the lotic ecosystems. Although microbial ecology of river and stream ecosystems is up to now a less intensively investigated field (Leff 1994), microbiological investigations of lotic systems revealed the important role of the complex microbial river communities in global nutrient cycles. It has become apparent that microbial transformations have a serious impact on the flux of organic carbon in running waters (Allan 1995, Schwoerbel 1999). This microbial loop (Azam et al. 1982) is of considerable significance not only in streams and rivers (Allan 1995) but also in lakes and marine systems. As a consequence, scientific attention is nowadays focusing on the role of bacterial production and the microbial food web as well as their impact on the self-purification capability of lotic systems. In river systems there are not only free-swimming planktonic bacteria, but also bacteria which are attached to the mobile interfaces of aquatic aggregates (mobile biofilms). Together with algae, fungi, protozoa and detritus, these bacteria constitute a major part of the mixed assemblages present in aquatic systems, with hot spots of high nutrient concentrations and microbial activity (Azam et al. 1993).

Aggregates are fragile microscopic particles of different forms and sizes, formed of suspended organic and inorganic matter in the water column and originate from combined biological and physical processes (Kiørboe et al. 1990). As they originate

from suspended matter, their composition varies temporally and spatially. Aggregate sizes range from a few microns to many centimeters, depending on the environment in which they occur (Alldredge & Silver, Grossart & Simon 1993, Zimmermann-Timm et al. 1998). The largest aggregates, larger than 0.5 mm in diameter occur in oceans, Alldredge & Silver (1988) described them as marine snow, composed of organic detritus, living photosynthetic and heterotrophic microorganisms and inorganic particles. Comparable aggregates but smaller in size (0.3 mm in diameter) were found in the pelagic zone of lakes (lake snow) (Paerl 1973, Grossart & Simon 1993). According to these common traits, we suggested the term “river snow” for the characteristic macroscopic aggregates in lotic systems (Böckelmann et al. 2000, Neu 2000). A common feature of aggregates is that they are rich in nutrients (Azam & Smith 1993), a fact that enhances their colonization by detrital communities of bacteria, phytoplankton, protozoans, and even metazoans. Studies on the bacterial assemblages within aggregates revealed that the bacterial content is two or three orders of magnitude greater than in the surrounding water (Davoll & Silver 1986, Grossart & Simon 1993). Since aggregates contain such an enriched microbial community, they are important sites in the water column for processes of photosynthesis, decomposition, and nutrient regeneration to occur at highly elevated levels (Alldredge & Silver 1988). Studies investigating the microbial community of lotic ecosystems so far can be divided into three different categories.

The first type of investigation is the description and characterization of the microbial community members by cultivation and molecular methods. The assemblage- or system-level approach includes measuring of cell density, biomass and viable counts based on respiratory activity or the ability to form colonies on solid media (Leff 1994, Leff et al. 1998). However, microbiologists are aware that conventional cultivation methods cannot provide a representative image of the true composition of microbial communities (Roszak & Colwell 1987, Staley & Konopka

1985), and in most cases less than 1% of the natural bacteria population in streams could be cultured (Leff 1994). Most of these microorganisms are oligothrophic, used to low nutrient conditions in their natural habitat and low nutrient media should be preferred. Wagner et al. (1993) showed that the use of rich media for the cultivation of the bacterial community in activated sludge favoured the growth of the in situ non-relevant gamma-*Proteobacteria*. Nevertheless, the cultivation method offers the possibility of physiological and morphological investigations with the isolated bacterial strains. Autecological approaches using modern molecular techniques, including the extraction and analysis of DNA and RNA, PCR, gene clone libraries, ARDRA (amplified ribosomal DNA restriction analysis), RFLP (restriction fragment length polymorphism) and fluorescent in situ hybridization (FISH) have become widely applied methods to identify bacteria without prior cultivation within their natural habitats. For example Hahn et al. (1992) investigated bacterial communities in soil, whereas Kalmbach et al. (1997a, 1997b), and Manz et al. (1993) identified bacteria in drinking water and lotic biofilms. Alfreider et al. (1996) and Grossart & Simon (1993) analysed bacterial assemblages in oligotrophic lakes and the bacterial diversity of marine systems was investigated for example by Dang & Lovell (2000), Matthew et al. (2000), Ramsing et al. (1996) and Ravenschlag et al. (2000). Bacterial communities of activated sludge were investigated by Manz et al. (1994, 1996, 1998) and Wagner et al. (1993). Nevertheless investigations of bacterial communities of running waters are rare. Recently, the overall structure and bacterial composition of specific lotic biofilm communities have been described (Manz et al. 1999). In another study, planktonic bacteria of the Japanese rivers Minoh and Neya were investigated, as one distinct compartment of the lotic community (Kenzaka et al. 1998). Leff and coworkers investigated the seasonal changes in planktonic bacterial assemblages of two Ohio streams by measuring the abundance of total bacteria, colony forming units and the population of *Pseudomonas cepacia* by hybridization with a species-specific

probe (Leff et al. 1998).

The second type of study, a structural investigation considered not only cellular but also non-cellular components of the aggregates for example the extracellular polymeric substances (EPS), which form the biofilm matrices. They are difficult to isolate and their chemical characterization is restricted and unable to provide integral information of the whole community structure (Nielsen & Jahn 1999). The use of antibodies for the EPS characterization is a suitable method for pure cultures. In environmental samples, in which large parts of the glycoconjugates are still unknown the use of polyvalent antibodies against EPS mixtures might be successful. Nevertheless, due to the significance of EPS, with regard to structure and function of biofilms, in situ approaches similar to in situ hybridization are strongly desirable. Decho & Kawaguchi (1999) examined extracellular polymeric secretions of microbial communities in marine stromatolite sediments after embedding them in Nanoplast and staining with FITC-labelled lectin from *Canavalia ensiformis*. Leriche et al. (2000) used an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. Recently lectin-binding-analysis with FITC-labelled lectins was introduced as a promising technique to visualize glycoconjugates in non-fixed, fully hydrated biofilm systems (Lawrence et al. 1998, Michael & Smith 1995, Neu 2000, Neu & Lawrence 1999a, 1999b). In contrast to previous descriptions, lectins are nowadays discussed as proteins with both a lectin-carbohydrate and a lectin-protein-binding site and are characterized on the basis of the interaction with certain mono- or oligo-saccharide residues. On a more cellular level, FITC-labelled lectins were used for the detection of baterial slime produced by *Staphylococcus epidermidis* (Sanford et al. 1995), visualization of algal and bacterial exopolymers (Grossart 1999), specific staining of Gram-positive, acidophilic mining bacteria (Fife et al. 2000) and the detection and quantification of glycoconjugate clusters within complex microbial biofilms (Neu & Lawrence 1999b). Structural analysis result in the

description of the spatial distribution of specific glycoconjugates originating from different eucaryotic and procaryotic microorganisms in lotic microbial aggregates. An additional prerequisite for structural investigations of microbial communities is the conservation of the native state of the environmental sample. One possible strategy to investigate microorganisms within their natural spatial distribution is the embedding and sectioning of the samples prior to further treatment. Conventional embedding procedures in agarose (MacNaughton et al. 1996), paraffin wax (Licht et al. 1996, Poulsen et al. 1994, Rothemund et al. 1996), and hard setting resins (2-hydroxyethyl-methacrylate) (Christensen et al. 1999, Gerrits & Smid 1983) are suitable methods for the conservation of compact structures, for example marine sponges (Manz et al. 2000), but are less suitable for the more fragile aquatic aggregates.

The third type of investigation deals with the function and the activity of the various members of the microbial community. This study is based on the results of the first and second study. Moreover it is trying to explain the effects of environmental change on aquatic microbial communities. Bacteria of lotic organic aggregates are involved in multifacetted interactions between the various eucaryotic and procaryotic inhabitants. The idea that most of these interactions are competitive has been invoked routinely for more than half a century focusing on the eucaryotic organisms (Allan 1995). Protozoans, such as flagellates and ciliates, are able to influence the microbial communities of aggregates by grazing on the bacterial assemblage (Güde 1996). Nevertheless, the bacteria also developed contrasting strategies in coexistence with a predator (Pernthaler et al. 1997) or living in dense association with each other in biofilms or as colonies on surfaces (Lawrence et al. 1995). Communication between the cells and a kind of “social behaviour” might be a useful and thinkable survival strategy. Moreover microbiologists concerning with bacterial colonization of biological and nonbiological surfaces, with mating, with development,

with biodegradation and with host-parasite relationships are being forced to consider the mechanisms whereby bacterial cells may communicate with each other (Dworkin 1991). Microbial cell-cell interactions are divided into those that are dynamic and involve the exchange of information between the cells, and those, which have the function to establish and maintain a structural, multicellular matrix (Dworkin 1991). One type of signal molecules, the autoinducers N-acetyl-homoserine lactones (AHLs) were detected in a variety of Gram-positive and Gram-negative bacteria (for example Geiger 1994, Pearson et al. 1994, Stickler et al. 1997) in equal concentration in the cytoplasm and in the surrounding medium. If autoinducers surpass a certain threshold value in dense bacterial cultures, genes are switched on in all cells of the autoinducer-producing strain. Recent studies have indicated the presence and importance of AHL systems in biofilm communities (McLean et al. 1997; Davies et al. 1998).

The aim of the present study was a comprehensive characterization of the bacterial community of river snow obtained from the river Elbe using traditional cultivation techniques, molecular methods and structural investigations. Cultivated bacteria were further characterized by phylogenetic analysis, proof of in situ relevance and their physiological abilities. Based on the results of the optimized cultivation strategy, bacteria of the river snow community of the South Saskatchewan river in Canada were cultivated. This led to the isolation of the bacterial strain F8 noticed by a remarkable kind of cell-cell interaction, which was further investigated.

2. Material and methods

2.1. Sampling of river snow

River snow samples were collected from five different rivers. The majority of the investigations of this study were performed with river snow samples collected from the Elbe River. Compared investigations were done with river snow samples from the South Saskatchewan River, the Oder River, the Rhine River and the Spree River. The river Elbe (Germany) is one of the large river systems in middle Europe, characterized by a discharge of 379 m^3 s^{-1}, a particulate load of 24.2 mg l^1, TOC (total organic carbon) 8.4 mg l^{-1}, TIN (total inorganic nitrogen) 3.97 mg l^{-1}, total P 0.24 mg l^{-1}, COD (chemical oxygen demand) 23.5 mg l^{-1} and a saprobic index of 2.13 (all mean values of 12 month of the year) (Landesamt für Umweltschutz Sachsen-Anhalt 2000). For each sampling a total of 10 l Elbe river water was used. Samples were collected in 10 separate glass bottles (1 l volume each) 0.5 m below the water surface at km 322 of the right downstream Elbe bank near Magdeburg. Lotic microbial aggregates from the South Saskatchewan River were obtained from the right downstream bank at Saskatoon (Saskatchewan, Canada). Samples of river snow from the Rhine River were collected at km 378 of the right downstream bank near Düsseldorf and river snow samples from the Oder River were obtained the right downstream bank at Eisenhüttenstadt. Spree River samples were collected at the right downstream bank near the Marchbridge in Berlin. Initially, the samples were transported within 30 min to the laboratory. After this time most of the aggregates were sedimented at the bottom of the flasks. In order to avoid influence of planktonic bacteria from the free water column, the major part of the river water was discharged. To further concentrate the particulate river snow aggregates, all individual (10 x 1 l) samples were centrifuged (13,000 x g) for 5 min and resuspended in 10 ml sterile (0.2-µm-pore-size filtered) river water each. In preparation for in situ hybridization

1 ml aliquots of each sample were subsequently fixed with 1 ml 3.7% (v/v) formaldehyde solution and incubated for 1.5 h at 4°C. Subsequently samples were washed with PBS, resuspended in a 1:1 mixture of PBS with ethanol and stored at -20°C as described by Manz et al. (1992). Sampling of Elbe river snow was repeated 3 times in weekly intervals in spring, summer autumn and winter 1998, respectively, Lotic microbial aggregates from the Rhine River, the Oder River and the Spree River were collected in June 1998. Sampling of river snow from the South Saskatchewan River at Saskatoon, Saskatchewan, Canada was performed in July 1999. For structural investigations by fluorescent in situ hybridization and lectin-binding-analysis (FISH-LBA) lotic microbial aggregates from the Elbe River were collected directly into coverslip chambers (Nunc, Roskilde, Denmark), which were immediately covered with a lid, sealed with parafilm and transported within 30 min to the laboratory. During sample treatment the samples were kept at room temperature in the dark. The complete subsequent treatment of the aggregates was performed within the coverslip chambers.

2.2. *Cultivation of river snow bacteria*

For the cultivation of river snow bacteria 10 x 1 l samples were centrifuged (13,000 x g) for 5 min and resuspended in 10 ml sterile (0.2-µm-pore-size filtered) river water each, pooled and used as inoculum (river snow suspension). Cultivation was performed immediately after sampling with a variety of aerobic and anaerobic media to obtain maximum cultivation efficiency.

2.2.1. *Enrichment and isolation of aerobic river snow bacteria from the Elbe*

A total of 13 different liquid and solid media (Table 1) were used for the

enrichment and isolation of aerobic river snow bacteria. Serial dilutions of the bacterial river snow suspension were plated directly on R2A agar, R2A agar supplemented with casitone (Difco), MacConkey agar (Difco), Endo agar (Difco) and freshwater basal medium (FBM). FBM media were in part further supplemented with yeast extract or yeast extract plus glucose. All enrichment cultures were incubated at 26°C in the dark. For the cultivation of *Planctomycetales* and other oligothrophic bacteria, river snow samples were pre-incubated in sterile Elbe raw river water without supplements, or supplemented with (i) peptone, (ii) peptone plus yeast extract, (iii) N-acetylglucosamine or (iv) chitin, and incubated aerobically at room temperature in the daylight (Schlesner 1994). Serial dilutions were plated weekly on PYGV-agar containing 20 ml Hutner´s salts solution (Cohen-Bazire et al. 1957) and 10 ml vitamin solution (Staley 1968). Different bacterial colonies were transferred on the corresponding solid media until pure cultures could be observed by uniform colony- and cell- morphology. In order to increase the yield of biomass, those bacterial isolates, which were characterized by colonies of pin-point size, were transferred to the corresponding liquid medium. They were incubated at 26°C on a rotary shaker, harvested after 1 to 7 days of incubation and fixed as described previously (Manz et al. 1992). All aerobic river snow isolates were subjected to physiological characterization using the commercial API-E and API-NE system (BioMerieux Deutschland, Nürtingen, Germany).

TABLE 1. Composition of the media used for the isolation of aerobic and anaerobic Elbe river snow bacteria

Medium	Composition per liter deionized water	Used for the isolation of
[a] RRW	natural composition	Oligothrophic bacteria
[a] RRW1	RRW supplemented with peptone (0.1% w/v)	Oligothrophic bacteria
[a] RRW2	RRW supplemented with peptone (0.005% w/v) plus yeast extract (0.005% w/v)	Oligothrophic bacteria
[a] RRW3	RRW supplemented with N-acetylglucosamine (0.1% w/v)	Oligothrophic bacteria
[a] RRW4	RRW supplemented with chitin (0.1% w/v)	Oligothrophic bacteria
[b] FBM	3.0 g Na_2SO_4, 0.4 g $MgCl_2$ x $6H_2O$, 1.2 g NaCl, 0.3 g NH_4Cl, 0.15 g $CaCl_2$ x $2H_2O$.	Oligothrophic bacteria
[b] FBM1	FBM supplemented with yeast extract (0.01% w/v)	Oligothrophic bacteria
[b] FBM2	FBM supplemented with yeast extract (0.01% w/v) plus glucose (0.5 mM)	Oligothrophic bacteria
PYGV	peptone (0.025% w/v), yeast extract (0.025% w/v), glucose (0.025% w/v), 20 ml Hutner´s salts solution, 10 ml vitamin solution	Oligothrophic bacteria, *Planctomycetales*
R2A	0.5 g yeast extract, 0.5 g Difco proteose peptone no. 3, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g sodiumpyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4$ x $7H_2O$	Freshwater bacteria
R2A1	supplemented with 3 g casitone (Difco)	Freshwater bacteria
Endo agar (Difco)	10.0 g bacto peptone, 10.0 g bacto lactose, 3.5 g dipotassium phosphate, 15.0 g bacto agar, 0.5 g bacto basic fuchsin, 2.5 g sodium sulfide	Enterobacteriaceae
MacConkey agar (Difco)	17.0 g bacto peptone, 3.0 g proteose peptone, 10.0 g bacto lactose, 1.5 g bacto bile salts no. 3, 5.0 g sodium chloride, 13.5 g bacto agar, 0.03 g bacto neutral red, 0.001 g bacto crystal violet	Enterobacteriaceae
[c] AMM1	detailed composition see Tschech & Pfennig (1984), supplemented with ethanol, or acetate, or benzoate, or formate, or pyruvate or propionate (10 mM each), plus Na_2SO_4 each (10 mM)	Sulfate-reducing bacteria (SRB)
[c] AMM2	supplemented with acetate (20 mM), or formate (20 mM), or methanol (5 mM)	Methanogenic bacteria
[c] AMM3	supplemented with pyruvate (20 mM), or glucose (20 mM), or casamino acids (0.5% w/v), or starch (0.5% w/v), or pectin (0.5% w/v), or cellulose (0.5% w/v), or chitin (0.1%)	Fermentative bacteria
[d] RCM agar (Merck)	10.0 g meat extract, 10.0 g casein peptone, 3.0 g yeast extract, 5.0 g glucose, 1.0 g starch, 5.0 g NaCl, 3.0 g sodium acetate, 0.5 g L-cysteine HCl x H_2O, 12.5 g agar	Clostridia
Brewer agar (Merck)	10.0 g proteose peptone no. 3, 10.0 g glucose, 5.0 g pancreatic digest of casein, 5.0 g yeast extract, 5.0 g NaCl, 2.0 g sodium thioglycolate, 1.0 g sodium formaldehyde sulfoxylate, 2.0 mg resazurin	Clostridia

[a] RRW: raw river water, **[b]** FBM: freshwater basal medium; **[c]** AMM: anaerobic mineral medium; **[d]** RCM: reinforced clostridial agar

2.2.2. *Cultivation of aerobic Elbe batch cultures*

100 ml sterile Elbe raw river water without supplements, or supplemented with (i) peptone, (ii) peptone plus yeast extract, (iii) N-acetylglucosamine or (iv) chitin, (see Table 1) were inoculated with 1 ml river snow suspension and incubated for 2 weeks aerobically at room temperature in the daylight (Schlesner 1994). At the beginning of the experiment, after 1 week, and at the end of the experiment, after 2 weeks, 1 ml samples were fixed with 1 ml 3.7% (v/v) formaldehyde solution and stored at –20°C as described in 2.1.

2.2.3. *Enrichment and isolation of anaerobic river snow bacteria from the Elbe*

For the enrichment and cultivation of anaerobic bacteria an anoxic, bicarbonate-buffered, sulfide reduced mineral medium (AMM) (Tschech & Pfennig 1984), supplemented with vitamin solution (Pfennig 1978) was used. To obtain a broad range of different anaerobic bacteria, basal media were supplemented with specific substrates, as summarized in Table 1. 100 ml butyl-rubber-sealed serum bottles, containing 50 ml of medium were each inoculated with 1 ml river snow suspension. For the enrichment of sulfate-reducing bacteria media were supplemented with lactate, ethanol, acetate, benzoate, formate, pyruvate or propionate. Na_2SO_4 was added to all enrichment assays at a final concentration of 10 mM. Methanogenic bacteria were enriched by the addition of acetate, formate, or methanol. For the enrichment of fermentative bacteria, pyruvate, glucose, casamino acids, starch, pectin, cellulose or chitin served as carbon source, and no further electron acceptor was added.

Anaerobic cultivation was performed under N_2-CO_2 (90:10 v/v) atmosphere. Bacteria grown in enrichment cultures were transferred in deep agar dilution series

using the media as described above. Sulfate-reducing bacteria were incubated at 26°C in the dark, and determined by the formation of black iron sulfide precipitates. Isolates obtained from enrichment cultures under methanogenic conditions were incubated at 37°C in the dark. After several passages in deep agar dilution each pure culture was transferred into 50 ml butyl-rubber-sealed serum bottles, containing 25 ml of the corresponding medium. Transfers were made with N_2-flushed syringes. Fermentative bacteria were plated in serial dilutions on RCM and Brewer agar (Merck, Darmstadt, Germany) and incubated for 1 to 7 days at room temperature in an anaerobic jar using the Anaerocult A system (Merck, Darmstadt, Germany). Colonies of different morphologies were transferred to liquid media as described above and fixed as described previously (Manz et al. 1992).

2.2.4. *Isolation of strain F8 from the South Saskatchewan River*

Serial dilutions of the bacterial river snow suspension were plated directly on three different oligotrophic solid media. Sterile river water (0.2-µm-pore-size filtered) supplemented with 100 µl l^{1} glucose stock solution (20 g l^{-1}); sterile river water (0.2-µm-pore-size filtered) supplemented with 100 µl l^{-1} each glucose-, ammonium chloride (45 g l^{1})- and phosphate (11 g l^{1})-stock solutions and freshwater basal medium FBM were used. FBM medium (detailed composition see Table 1) was supplemented with yeast extract (0.01% w/v) and glucose (0.5 mM). Agar plates were incubated at room temperature in the dark for a period of 7 days. Only bacterial colonies of striking morphology, which were mostly transparent and of pin-point size or even smaller, were transferred to the corresponding solid and liquid medium. After several passages the cell morphology of all isolated pure bacterial cultures (in total 36) was examined microscopically. Microscopical investigation led to the detection of isolate F8, which was characterized by the formation of a filamentous network.

2.3. *Determination of total cell counts (DAPI and SYTO9 staining)*

For the determination of the total bacterial cell counts 1 ml river snow samples were sonificated for 10 min (Branson Sonifier, Danbury, USA) to obtain a uniform distribution of the cells. Samples were subsequently diluted 1:10 and filtered through polycarbonate membranes (0.2-µm-pore-size, Millipore, Eschborn, Germany). After air drying, the membranes were mounted on glass slides with 2 µl DAPI solution (4',6-diamidino-2-phenylindole, Sigma, Deisenhofen, Germany, 1 µg ml^{-1}) and stained with additional 10 µl DAPI solution for at least 15 min (absorption maximum 358 nm, emission maximum 461 nm). Prior to microscopical analysis, anti-fading reagent (Citifluor AF2, Citifluor Ltd., London, UK) was added. At least 10 microscopic fields (100 by 100 µm) were chosen randomly and a minimum of 1,000 cells were enumerated microscopically. For the FISH-LBA method DAPI was applied to fixed river snow samples and SYTO9 was used for staining of unfixed, fully hydrated, living river snow samples at a final concentration of 20 µg ml^{-1} for 5 min (absorption maximum 480 nm, emission maximum 500 nm). For this study both stains were applied without prior sonification of the sample in order to avoid destruction of the aggregates.

2.4. *Fluorescent in situ hybridization of river snow bacteria*

2.4.1. *Oligonucleotide probes*

The sequences, references and target organisms of the rRNA oligonucleotide probes used in this study are summarized in Table 2. Custom synthesized oligonucleotides were 5'-labelled with CY3 (Metabion, Planegg, Germany).

Additionally, unlabelled oligonucleotides GAM42a, BET42a and beta8b were used for competitive hybridizations with probes BET42a, GAM42a and beta8a as described by Kalmbach et al. (1997b). All oligonucleotides were stored in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) at -20°C. Working solutions were adjusted to 50 ng DNA μl^{-1}.

TABLE 2. Oligonucleotide probes, target-organisms and stringencies used for river snow community analysis
(FA = percentage formamide in hybridization buffer)

Probe [a, b]	Target-organisms	FA (%)	[NaCl] (mM)	Reference
S-D-Bact-0338-a-A-18 (EUB338)	Domain *Bacteria*	20	250	Amann et al. 1990
S-D-Bact-0338-a-S-18 (non-EUB338)	serves as negative control	20	250	Amann et al. 1990
S-Sc-aProt-0019-a-A-17 (ALF1b)	α-subclass of *Proteobacteria*	35	88	Manz et al. 1992
L-Sc-bProt-1027-a-A-17 (BETA42a)	β-subclass of *Proteobacteria*	35	88	Manz et al. 1992
S-St-IsoB8-0069-a-A-19 (beta8b)	*Aquabacterium commune*	50	31.2	Kalmbach et al. 1997b
L-Sc-gProt-1027-a-A-17 (GAM42)	γ-subclass of *Proteobacteria*	35	88	Manz et al. 1992
S-P-CyFla-0319-a-A-18 (CF319a)	*Cytophaga-Flavobacteria*	35	88	Manz et al. 1996
S-P-CyFla-0319-b-A-18 (CF319b)	*Cytophaga-Flavobacteria*	35	88	Manz et al. 1996
S-P-Pla-0046-a-A-18 (PLA46)	*Planctomycetales*	35	88	Neef et al. 1998
S-P-HGC-1901-a-A-18 (HGC69a)	Gram-positive bacteria with a high GC content of DNA	35	88	Roller et al. 1994
S-F-Leg-0705-a-A-18 (LEG705)	*Legionellaceae*	35	88	Manz et al. 1994
S-P-Bac-0303-a-A-17 (BAC303)	Bacteroides cluster of phylum cytophaga-flavobacter-bacteroides	20	250	Manz et al. 1996
S-F-Srb-0385-b-A-18 (SRB385Db)	Most members of delta subclass of *Proteobacteria* including *Desulfobacteriaceae*	35	88	Rabus et al. 1996
S-D-Arch-0915-a-A-20 (ARCH915)	Domain *Archaea*	20	250	Stahl & Amann 1991
S-F-M.sa.-0860-a-A-21 (MSMX860)	*Methanosarcinaceae*	35	88	Raskin et al. 1994

[a] *Escherichia coli* numbering (Brosius et al. 1981); [b] probe nomenclature as described by Alm et al. (1996). All oligonucleotide probes are 16S rRNA probes with the exception of BETA42a and GAM42 which are 23S rRNA probes.

2.4.2. *Hybridization procedure*

Hybridizations of fixed samples were performed in a humid chamber at 46°C for 1.5 to 4 h as described by Manz et al. (1994). The hybridization solution consisted of 5 ng μl^{-1} oligonucleotide probe, 0.9 M NaCl, 20 mM Tris/HCl (pH 8), 0.01% SDS, and the appropriate amount of formamide as given in Table 2. The slides were washed by immersion for 20 min in 46°C washing solution containing 20 mM Tris/HCl and 0.01% SDS. The NaCl-concentration of the washing solution corresponded to the formamide concentration of the hybridization solution and is given in Table 2. The determination of total cell counts was performed by staining with DAPI without prior sonification. Individual river snow samples (n = 10) were microscopically examined, counting cells in at least 10 randomly chosen microscopic fields (100 by 100 µm) and a minimum of 1,000 cells for each sample. The microscopic field was first viewed with the CY3 filter set. After this, the filter settings were changed to the DAPI filter set, to avoid bleaching of the fluorochrome CY3 during DAPI examination. Probe specific cell counts were given as the percentages of total cell counts, which were determined by DAPI staining and separately calculated for each microscopic field (replicate, n = 10).

2.5. *Statistical evaluation*

With each replicate statistical analysis of each group of specific hybridization values was done with the StatsDirect software package (CamCode, Ashwell, Herts, UK). Differences between specific cell counts in spring, summer, autumn, and winter were analysed using one way ANOVA, followed by multiple mean comparisons of the Bonferroni test for selected pairs, and finally the Tukey-Kramer test was applied to calculate each pair against all others.

2.6. *Fluorescent in situ hybridization of the undestroyed aggregates*

The complete procedure was performed in 8-well coverslip chambers (Nunc, Roskilde, Denmark) with a sample volume of 100 µl per well. After every step of the treatment, the liquid component of the sample was absorbed carefully using filtertips leaving always about 50 µl liquid in each well to prevent drying up of the sample. All lectin-staining- and hybridization-steps were performed in the dark at room temperature and 46 °C, respectively. For in situ hybridization, the aggregates were fixed by the addition of 200 µl formaldehyde solution (3.7%) for 30 min at 4 °C, and subsequent removal of the formaldehyde solution with filtertips. After this, samples were overlayed with 100 µl of increasing concentrations of ethanol (50%, 80%, 96%, v/v) for 3 min each. The ethanol solution was absorbed using filtertips after each step. Probe EUB338, specific for the majority of Bacteria (Amann et al. 1990), and probe BETA42a, specific for the beta-subclass of *Proteobacteria* (Manz et al. 1992) were used for hybridization. Unlabelled oligonucleotide probe GAM42a (Manz et al. 1992) was used for competitive hybridization with probe BET42a, as described by Manz et al. (1992). All custom synthesized oligonucleotide probes were 5'-labelled with the indocarbocyanine dye Cy3 (Metabion, Planegg, Germany) and stored in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) at –20 °C. Hybridization solutions were adjusted to a final concentrations of 5 ng probe μl^{-1}. The prewarmed (46 °C) hybridization solution (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, 0.01% SDS, 35% (v/v) formamide) was mixed in a ratio of 9:1 with the fluorescently labelled oligonucleotide. To each well of the coverslip chamber 100 µl hybridization solution was added to the sample and incubated for at least 90 min at 46 °C in the dark for hybridization. To prevent evaporation the coverslip chamber was covered with a lid and sealed with parafilm. Unbound oligonucleotides were carefully removed by two washing steps with 500 µl pre-warmed washing buffer (20 mM Tris/HCl, 0.01% SDS, 88 mM NaCl)

for 20 min and 5 min, respectively.

2.7. *Lectin-binding-analysis*

With the exception of the lectin originating from *Pseudomonas aeruginosa*, all lectins used in this study were commercially labelled with FITC or TRITC (Sigma, USA). The *Pseudomonas aeruginosa* lectin (Sigma, USA) was labelled with the FluoroTagTMFITC Conjugation Kit (Sigma, USA) according to the manufacturer`s instructions. Each freeze-dried lectin was diluted in distilled water in concentrations ranging from 1 to 10 to 100 ng μl^{-1} to find out the optimal lectin concentration for staining. The otimal lectin working concentration was determined microscopically and defined as strong fluorescent specific binding signals in river snow samples without unspecific background staining. Lectin source, the common name, the known sugar-specificity and the working concentration of all lectins used in this study are summarized in Table 3. After in situ hybridization, the river snow samples were incubated in the coverslip chambers with 100 µl lectin solution, and incubated for 20 min at room temperature in the dark. To remove unbound lectins from the samples, the aggregates were carefully washed four times with 500 – 1000 µl washing buffer as described above. Samples could be stored at –20 °C for several weeks without remarkable loss of probe- or lectin conferred fluorescence intensity.

TABLE 3. Characteristics of lectins employed in this study
Data are taken from the material data sheet of the supplier

Lectin source	Common name (abbreviation)	Sugar specificity	Concentration used (ng μl^{-1})
Arachis hypogaea	Peanut (PNA)	D (+) galactose α lactose	100
Limulus polyphemus	Horseshoe crab (Limulin)	N-acetylneuraminic acid D-glucuronic acid	10-50
Phaseolus vulgaris	Red Kidney bean (PHA)	none of the tested sugars	50
Pseudomonas aeruginosa	*Pseudomonas aeruginosa* (PA-I)	D (+) galactose N-acetylgalactosamine	1-10
Triticum vulgaris	Wheat germ (WGA)	NN' diacetylchitobiose NN'N''-triacetylchitobiose	50-100

2.8. *Lectin blocking assays*

Controls for the specificity of the lectin-binding were performed with *Limulus polyphemus* lectin, in combination with the lectin-specific carbohydrates D-glucuronic acid (Sigma, USA), and N-acetylneuraminic acid (Sigma, USA), and the bacterial Elbe river snow isolate 18 described by Böckelmann et al. (2000). A stock solution of the carbohydrates was prepared at a concentration of 10 mg ml^{-1} D-glucuronic acid and 1 mg ml^{-1} N-acetylneuraminic acid each in 30 mM phosphate buffered saline, pH 7.2. These concentrations as well as a decadal dilution series from 1:10 to 1:10000 were used for the competitive-binding experiments. The lectin was applied at the optimal working concentration as for staining, and pre-incubated for 15 min with the corresponding carbohydrate at various dilutions before staining the bacterial isolate. The procedure applied for inhibition experiments was identical to the lectin staining described above.

2.9. *Characterization of the isolated river snow bacteria*

2.9.1. *Extraction of genomic DNA and amplification of the 16S rRNA genes*

Genomic DNA was isolated from bacteria grown on solid media (aerobic and facultative anaerobic isolates) or liquid media (anaerobic isolates) by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 16S rDNA sequences were amplified with the universal primers 8F (5'-AGAGTTTGATYMTGGCTCAG-3'), 1492R (5'-CGGYTACCTTGTTACGAC-3') (Lane 1991), 63F (5'-CAGGCCTAACACATGCAAGTC-3'), and 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Lane 1991). PCR mixtures contained 200 µM of each deoxynucleotide, 3 mM magnesium chloride, PCR buffer (10 mM Tris/HCl, 50 mM KCl, pH 8), 20 pM of each primer, 100 to 125 ng of genomic DNA, and 2.5 U *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). The polymerase chain reaction was performed using a Personal cycler (Biometra, Göttingen, Germany). The samples were 90 s subjected to an initial denaturation step at 96°C, followed by the addition of 2.5 U *Taq* polymerase to each sample. The thermal profile consisted of 35 cycles of 30 s at 96°C, 90 s at 52°C, and 90 s at 72°C, with an increment of 5 s. The PCR products were purified with the QIAquick PCR purification kit (Qiagen).

2.9.2. *16S ribosomal DNA sequencing and phylogenetic analysis*

Cycle sequencing of 16S ribosomal DNA was done using the BigDye Terminator Ready Reaction Kit (PE Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. In addition to the PCR primers given above, the following oligonucleotides were used: 610VII (5'-ACCGCGGCTGCTGGCAC-3'), 610RII (5'-GTCCCAGCAGCCGCGGT-3'), and 699RII (5'-RGGGTTGCGCTCGTT-3') (W.

Ludwig, pers. commun.). DNA sequencing was performed in a Personal cycler (Biometra) according to the manufacturer's protocol. Sequences were generated with an ABI373-sequencer (PE Applied Biosystems) and analysed with the sequence analysis software version 3.3 (Sequenzierservice Meixner, Berlin, Germany). The phylogenetic analysis of the sequences was done with the ARB software package (Strunk & Ludwig 1995). The 16S rDNA sequences were aligned by using the Aligner-tool of the ARB software package and manually corrected according to primary and secondary structure similarities. Distance matrices were constructed from the aligned sequences and corrected for multiple base changes at single positions by the method of Jukes & Cantor (1969). Phylogenetic trees were reconstructed by the neighbor joining method of Saitou & Nei (1987).

2.9.3. *Proof of in situ relevance of the isolated river snow bacteria*

2.9.3.1. *Design and evaluation of specific oligonucleotide probes*

New strain, group or cluster specific oligonucleotide probes for different isolated river snow bacteria were designed and evaluated using the Probe-Design and Probe-Match tool of the ARB software package (Strunk & Ludwig 1995) as described previously (Manz et al. 1998). The database was scanned for probes specifically, i. e. other bacteria should be discriminated by at least one mismatch in the target region. Potential candidates for analytical in situ probes were compared with the ARB database by using the Probe-Match tool of the ARB software package to search for organisms with complete homologies within the target sequences. The newly developed probes were purchased CY3-labelled from Metabion (Planegg, Germany). Their sequences, target organisms and stringencies are summarized in Table 4.

TABLE 4. Newly developed oligonucleotide probes, target-organisms and stringencies used for in situ hybridization
(FA = percentage formamide in hybridization buffer)

Probe	Sequence 5` - 3`	Target organisms	FA (%)	[NaCl] (mM)
BETA18	GCT CGT ATA GCG CGA GGC	Elbe river snow isolate 18	35%	88
BETA14	CGT ATG CGG TAG CTG ACC	Elbe river snow isolate 14	35%	88
BETA11	GCC TTC TTG CTG CAC CCG	Elbe river snow isolate 11	35%	88
BETA34	GCC CAC CTA TAA GGG GCC	Elbe river snow isolate 34	35%	88
BETA21	CCC CCA CAA CTG GGC ACG	Elbe river snow isolate 21	40%	62.4
BETAP10	CCT AAT GTG CCA TCG GCC	Elbe river snow isolate P10	35%	88
BETAClu34gr	CGT TCC GTA CAA AAG CAG	Elbe river snow isolates 11, 34, P10 and relatives	35%	88
BETAClu34kl	CGG CCG CTC CGT CCG CGC	Elbe river snow isolates 11, 34, P10 and closest relatives	35%	88
BETAClu18kl	CCG ACA TCG GCC GCT CGT	Elbe river snow isolate 18 and closest relatives	35%	88
AERO1244	GCT TGC AGC CCT CTG TAC GCG	*Aeromonadaceae*	60%	15.6
FLAVO1004	GGT CTG TTT CCA AAC CGG	Elbe river snow isolate 8	50%	31.2
CLOBU1022	CCT GCC ACC GAA GTG GCT	*Clostridium butyricum*, Elbe river snow isolates 9.1, 9.2, 9.4	35%	88

All oligonucleotide probes are 16S rRNA directed probes

2.9.3.2. *Determination of hybridization stringencies*

The optimization of the hybridization stringency was performed as described by Manz et al. (1998). In situ hybridizations were performed as described above using target and non-target organisms displaying one to two mismatches within the target region. The hybridization stringency was adjusted by the stepwise addition of formamide to the hybridization buffer in concentration steps of 10%. The NaCl-concentration of the washing buffer was adjusted to the formamide concentration used in the hybridization buffer.

2.9.3.3. *In situ hybridization of samples of different rivers with the newly developed probes*

To proof the in situ relevance of the isolated river snow bacteria fixed river snow samples of Elbe batch cultures, the Elbe River of different seasons, as well as summer river snow samples from the Rhine, the Oder and the Sree River were hybridized with the newly developed probes. Hybridization procedure was performed as described in *2.4.2.*

2.9.4. *Physiological abilities of the isolated aerobic river snow bacteria*

The metabolic pattern of the aerobic river snow isolates affiliated to the alpha-, beta-and gamma-*Proteobacteria* was checked with the Biolog GN MicroPlate test system (MERLIN Bornheim-Hersel). Biolog MicroPlate tests the ability of a microorganism to metabolize or oxidize a preselected panel of 95 different carbon sources. The test yields a characteristic pattern of purple wells which constitutes a "Metabolic Fingerprint" of the capacities of the inoculated organism. Tetrazolium violet was used as a redox dye to colourimetrically indicate the utilization of the carbon sources. River snow isolates were either grown on Tryptic soy agar (TSA Difco) or R2A agar medium for a recommended incubation period of 4 – 18 hours. Cells were removed from the agar plate with a sterile swab and suspended in sterile saline to an approximate cell density of about 3×10^8 cells ml^{-1}. The bacterial density was checked by comparison with McFarland Standard suspensions of known opacity (BioMerieux Deutschland, Nürtingen, Germany). For the saline-sensitive beta strains a lowered saline concentration (as given in the FBM medium) was used. Each well of the MicroPlates was inoculated with 150 µl cell suspension and incubated at 28°C in

the dark for 24 hours. For documentation of the results, the plates were read visually every 4 hours and evaluated after 24 hours. Carbon utilization analysis of strain F8 was carried out at the National Water Research Institute in Saskatoon, Canada, where the commercial multiwell system Biolog (Biolog, Hayward, CA) was used to assess the potential range of carbon sources used by the isolate F8. Cells were cultured on FBM medium, resuspended in phosphate buffered saline to achieve appropriate optical density (of about 3 x 10^8 cells ml^{-1}) and inoculated into the multiwell plates according to the manufacturers instructions. Plates were read using a plate reader and Biolog software at intervals of 4 hours over 24 hours to determine the appearance of positive well respectively carbon utilization.

2.10. *Determination of filament formation dependent on media composition*

To check whether filament formation of strain F8 was dependent upon nutrient concentrations, strain F8 was cultivated on different rich and low nutrient solid and liquid media. The following media were used: Nutrient broth (NB, Difco, USA) 20 g l^{1}; Tryptic soy agar (TSA, Difco, USA); 10 g l^{-1} or alternative 1 g l^{1}; R2A (Difco, USA), conc; Czapek-Dox medium (CD, Difco, USA), conc.; Cytophaga and Flavobacteria medium (CF, Difco, USA), conc; freshwater basal medium (FBM), sterile raw river water (0.2-µm-pore-size filtered) of the South Saskatchewan river supplemented with 100 µl l^{1} glucose (20 g l^{1}) stock solution (RRW a); sterile river water (0.2-µm-pore-size filtered) of the South Saskatchewan river supplemented with 100 µl l^{1} each glucose-, ammonium chloride (45 g l^{1})- and phosphate (11 g l^{1})-stock solutions, (RRW b). Filament development and abundance was checked microscopically after staining with SYTO9 (Molecular Probes, Eugene, OR, USA) at a final concentration of 20 µg ml^{-1} for 5 min.

2.11. *Histochemical characterization of filaments*

In order to further assess the nature of the filaments a range of various dyes targeting different constituents were used to stain the bacterial cells and filaments. Crystalviolet, Methyleneblue, Carbolfuchsin, MethylRed, RutheniumRed, CongoRed and MalachiteGreen as well as ink were used as described in (Romeis 1989), tannic acid was used following the protocol of Helmbrook et al. (1989). Staining-results were examined by light microscopy. Fluorescent dyes as the lipid-stain NileRed, the protein-stains SYPROOrange, SYPRORed, BODIPY, and the DNA-stains SYTOXGreen, SYTO9, SYTO15, SYTO62 as well as SYTO63 (Molecular Probes, Eugene, Oregon) were applied to the filament cell suspensions and used according to the manufacturers instructions. In addition the fluorescent Ca^{2+} indicator conjugates dextrans10.000MW, and 70.000MW, were employed in order to determine surface charge characteristica of the cells and filaments. DAPI (4',6-diamidino-2-phenylindole, Sigma, Deisenhofen, Germany) was used in a concentration of 1 µg ml^{-1} and samples were stained for 5 min. Staining results were examined by epifluorescence or confocal laser scanning microscopy. The lectins employeded in this study: *Arachis hypogaea*, *Bandeiraea simplicifolia* (*BS-I*), *Caragana arborescens, Concanavalin A, Erythrina cristagalli, Glycine max, Lens culinaris, Limulus polyphemus, Lycopersicon esculentum, Pseudomonas aeruginosa* (*PA-I*), *Tetragonolobus purpureas, Triticum vulgaris, Ulex europaeus* (*UEA-I*), (*UEA-II*), *Vicia faba, Vicia villosa,* and *Wisteria floribunda,* (Sigma, St Louis, MI, USA) were FITC or TRITC labeled. They were added at a concentration of 0.1 mg ml^{-1} for a staining period of 20 min. Staining results were examined by confocal laser scanning microscopy.

2.12. *Microscopical analysis and documentation*

The microscopical investigations of this study were performed at different institutes in Berlin, Magdeburg and Saskatoon therefore, different microscopes were used. Phase contrast micrographs of fixed river snow samples were taken with a Zeiss Microscope Axiophot 2 (Zeiss, Oberkochen, Germany). FISH signals were detected by epifluorescence microscopy using a Zeiss Axioskop (Oberkochen, Germany) equipped with Zeiss light filter sets no. 01 for DAPI (excitation 365 nm, dichroic mirror 395 nm, suppression 397 nm), and HQ light filter 41007 (AF Analysentechnik, Tübingen, Germany) for CY3-labelled probes (excitation 535-550 nm, dichroic mirror 565 nm, suppression 610-675 nm). For the FISH-LBA method confocal laser scanning microscopy (CLSM) of lotic aggregates was performed with a TCS 4D (Leica, Heidelberg, Germany) attached to an inverted microscope and equipped with an argon-krypton laser. Aggregates were observed with 20 x 0.6 NA, 40 x 0.75 NA, 63 x 1.2 NA W CORR and 100 x 1.4 NA lenses. Reflection images were taken with RT 30/70 and polarisation filters. The microscope was used in the single or multi channel mode to record the reflection signal, nucleic acid stained bacteria, stained glycoconjugates, hybridized microorganisms and autofluorescence of phototrophic organisms. Reflection images were taken at 488 nm. For the detection of DAPI stained cells a UV laser was available (excitation 351 – 364 nm, emission filter BP 440). Settings intended for FITC (excitation = 488nm, emission filter = BP FITC) in the green channel and CY3 (excitation = 568nm, emission filter = BP600) in the red channel were employed for detection with lectins and FISH. The settings intended for CY5 (excitation = 647nm, emission filter = LP 665) in the far red channel were used to record the autofluorescence of chlorophyll containing organisms. For presentation of micrographs the standard software ScanWare Ver. 5.1A (Leica) was used. The images were printed with Photoshop (Adobe, Edinburgh, UK) on a UP-D8800 digital

colour printer (SONY, Japan). Filament abundance assays of strain F8 were carried out using several different microscopical techniques. Light microscopy and epifluorescence microscopy were used as described above. For examination with the fluorescent dye SYTO9 confocal laser scanning microscopy (CLSM) was performed with either the TCS 4D (Leica, Heidelberg, Germany) attached to an inverted microscope or a Bio-Rad MRC-1000/1024 attached to an upright microscope (Nikon Microphot SA). The light source used for CLSM was an argon-krypton laser with excitation lines at 488 nm, 576 nm and 647 nm. The system was controlled by the standard software ScanWare Ver. 5.1A (Leica, Heidelberg, Germany) or Bio-Rad operating software (Bio-Rad, Hemel Hempstead, UK). Filaments were observed with 20 x 0.6 NA, 40 x 0.75 NA, 63 x 1.2 NA W CORR and 100 x 1.4 NA lenses. The CLSM microscopes were run in single or multi channel mode and images were presented as described above. Liquid and solid overnight cultures of strain F8 on CF, R2A and FBM medium were used for transmission electron microscopy. Preparation for transmission electron microscopy was done as follows: For negative-staining, samples of bacterial suspensions were dropped onto Piloform-coated copper grids. The material was negatively stained with a mixture of phosphotungstate (2%, w/v) and bacitracin (0.005%, w/v) according to Wolf et al. (1993). Thin sections of bacterial cells from exponential-phase cultures were prepared as described by Wolf et al. (1993). Fixation of the cells was carried out with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for at least 16 h at 4°C. After washing with cacodylate buffer, the cells were post-fixed with 1.5% (w/v) osmium tetroxide plus 1.65% (w/v) potassium dichromate in 0.1 M cacodylate buffer for 1 h at room temperature, dehydrated in a graded ethanol series and embedded in LR White (Science Services). Thin sections were cut with a Reichert OM U3 ultramicrotome and post-stained for 6 min with 1% (w/v) lead citrate. Examination of the thin sections was performed with a Phillips 400 electron microscope.

3. Results

3.1. *Microscopical examination of the Elbe river snow throughout the year*

During all seasons, river snow samples were characterized by their brown colour and were generally composed of organic detritus, inorganic particles, such as sand and clay, as well as various bacterial and algal species. The structure and composition of the microbial river snow community showed seasonal dynamics. River snow aggregates obtained in spring were large, loosely arranged with typical macroscopic dimensions of 0.5 to 5 mm in diameter. The predominant bacterial morphotypes were long thin filaments (0.5 µm in width and up to 40 µm in length) as shown in Fig. 1A. The typical feature of summer river snow aggregates were large amounts of green algae (*Pediastrum* spp., *Scenedesmus* spp.), diatoms (*Melosira* sp., *Navicula* sp., *Asterionella sp*., *Tabellaria* sp.) and cyanobacteria (*Oscillatoria* spp., *Chroococcus* spp.), shown in Fig. 1B. The overall size of summer river snow aggregates was to the spring river snow, with average diameters of 0.5 to 5 mm. In autumn and winter, river snow aggregates were remarkably smaller and were characterized by the absence of algae and a more compact structure, with typical dimensions of 0.5 to 2 mm, shown in Fig. 1C and D. The total bacterial cell counts of the river snow community (determined with DAPI, see 2.3) ranged between 2.5×10^8 cells ml^{-1} in spring, 2.0×10^8 in summer, 1.2×10^8 in autumn and 1.4×10^8 in winter (all mean values).

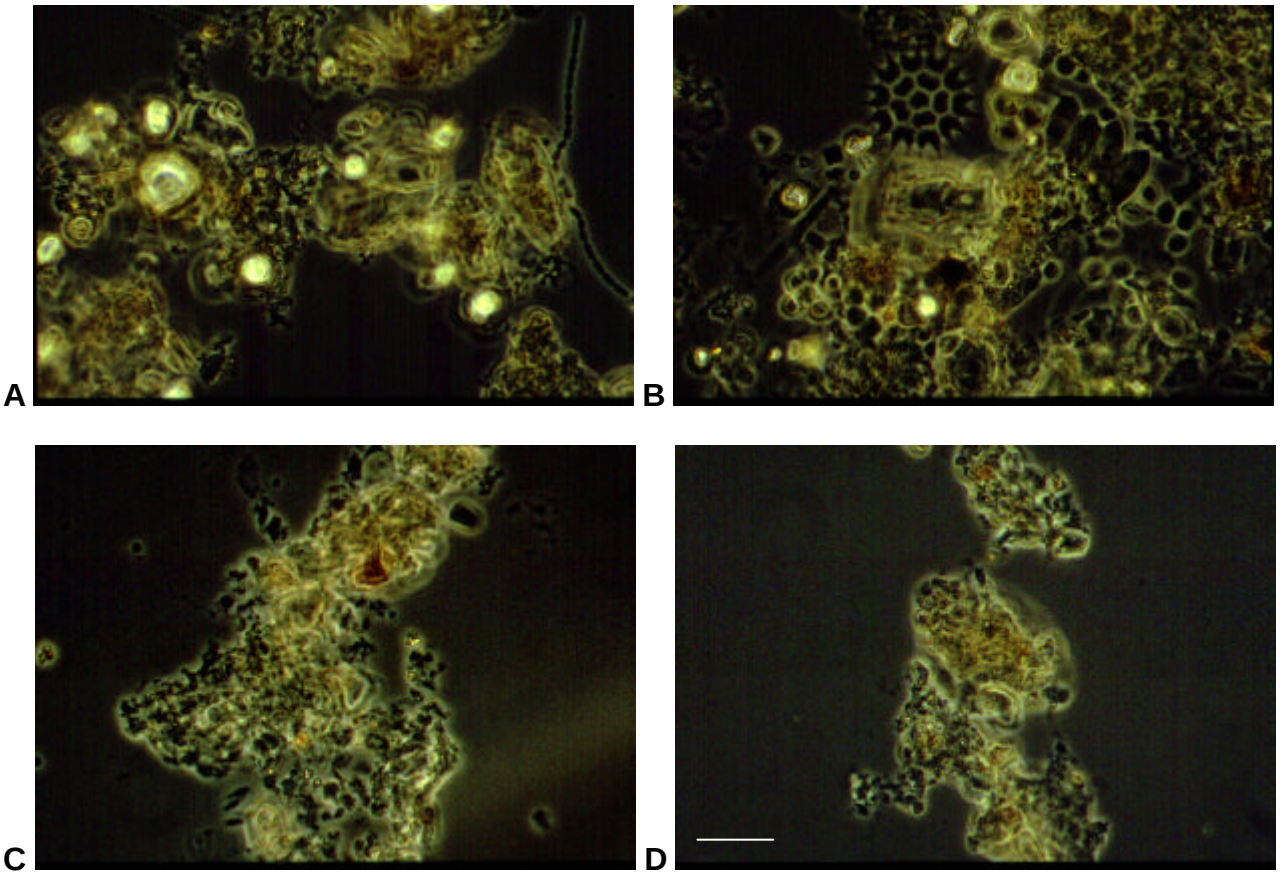


Fig. 1 Phase contrast photomicrographs of Elbe river snow samples obtained in spring **A**, in summer **B**, in autumn **C** and in winter **D**. Note that A and B show aereas of the large loosely arranged aggregates, whereas C and D show complete compact flocs. The micrographs were done at 400 x magnification, scale bar given in D equals 0.1 mm and is representative for all micrographs.

3.2. *Phylogenetic composition of the bacterial river snow community*

The relative in situ abundances of the main subclasses of bacterial groups within the river snow community collected in spring, summer, autumn and winter are shown in Fig. 2. The amount of DAPI stained cells which could be hybridized with the *Bacteria* probe EUB338 (Fig. 3A) varied from 79 ± 9% in spring and 75 ± 10% in summer to 74 ± 8% in autumn and 72 ± 9% in winter (n = 10) without significant seasonal differences between all four samples.

Throughout the year, beta-*Proteobacteria* constituted the numerically most important bacterial group. The relative abundance of beta-*Proteobacteria* ranged from 50 ± 10% to 54 ± 6% of the total cell counts (Fig. 2). In all river snow samples, up to 30% of the total cell counts, which equals to more than 50% of the beta-*Proteobacteria*, could be successfully hybridized with probe beta8b (Fig. 3B), recently developed for the detection of an in situ dominant bacterial isolate from drinking water (Kalmbach et al. 1997b). These short, rod shaped bacteria (1 µm in length) showed typical polyalkanoate inclusion bodies and they occurred as single cells or were arranged in short chains, or formed globular microcolonies within the river snow community. They were frequently observed tightly associated with different algae.

The amount of gamma-*Proteobacteria* ranged from 25 ± 8% of the total cell counts in spring, 21 ± 8% in summer, 14 ± 5% in autumn and 26 ± 8% in winter. The relative abundance of gamma-*Proteobacteria* was significantly different between autumn and spring (n = 10, p = 0.0255) and autumn and winter (n = 10, p = 0.0202). Typical morphotypes within the gamma-*Proteobacteria* were thick, rod shaped bacteria (1 µm in width and up to 3 µm in length), occurring as single cells or loosely arranged in small microcolonies.

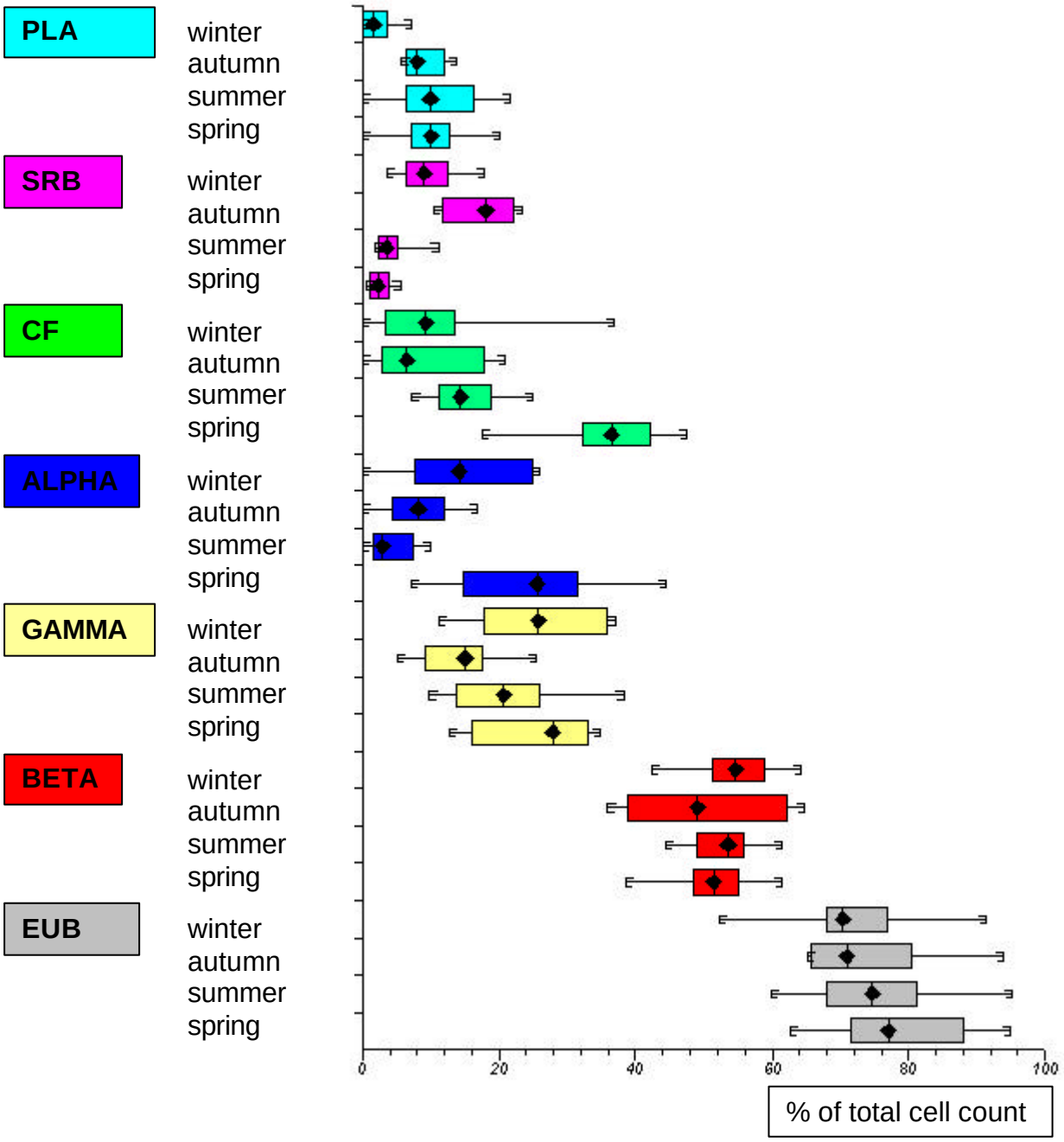


Fig. 2. Box-and-whisker plot of the seasonal distribution of the main phylogenetic bacterial groups within the microbial Elbe river snow community determined by FISH. Probe-specific cell counts are given as percentages of total cell counts determined by DAPI staining. The central box of each data set represents the distance between the first and the third quartiles. The median between them is marked with a diamond, with the minimum as the origin of the leading whisker and with the maximum as the limit of the trailing whisker.

Bacteria affiliated to the alpha-*Proteobacteria* constituted a minor part of the bacterial river snow population, with specific cell counts of 24 ± 10% in spring, 4 ± 3% in summer, 8 ± 5% in autumn, and 15 ± 8% in winter. As shown in Fig. 2, the relative abundance of alpha-*Proteobacteria* was significantly different between the microbial communities investigated in spring, relative to summer (n = 10, $p < 0.0001$), to autumn (n = 10, $p = 0.002$) and winter (n = 10, $p = 0.0361$), as well as the river snow community in summer, compared with the winter river snow (n = 10, $p = 0.0232$). The predominant members of the alpha-*Proteobacteria* were rod-shaped (0.5 x 2 µm) and irregularly formed coccoid cells (diameter 1 - 1.5 µm), typically growing in aggregates or in cell chains.

Bacteria detected with probe CF319a/b accounted for 15 ± 5% in summer, 9 ± 7% in autumn and 10 ± 10% in winter, and reached their highest level in spring with up to 36 ± 8% of the total cell counts. Statistical analysis confirmed these results indicating significant differences in the amount of *Cytophaga-Flavobacteria* in spring river snow relative to all other seasons (n = 10, $p < 0.0001$ each). In the spring river snow community, bacteria hybridizing with probe CF319a/b were mainly characterized by a long, thin, filamentous cell shape (up to 40 µm in length), forming a finely structured network throughout and around the river snow aggregates (Fig. 3C). In contrast to this, in river snow obtained in autumn and winter the number of filamentous *Cytophaga-Flavobacteria* was significantly decreased, but small, rod shaped cells (0.5 µm x 1 µm) could be hybridized with probe CF319a/b.

Sulfate-reducing bacteria, as visualized by hybridization with probe SRB385Db, constituted a minor part of the bacterial river snow community in spring and summer, with specific cell counts of 2 ± 1% and 4 ± 2%, respectively. The increase of SRB in winter 9 ± 4% and autumn 17 ± 4% outlined the seasonal distribution of this subpopulation. As depicted in Fig. 2, the amount of SRB in autumn river snow was

significantly different in contrast to all other seasons (n = 10, $p < 0.0001$ for autumn river snow in comparison to spring and summer, and $p = 0.0007$ for autumn river snow in comparison to winter). Significant seasonal variations could also be shown between winter and spring (n = 10, $p = 0.0012$), as well as winter and summer river snow (n = 10, $p = 0.00175$). Members of the sulfate-reducing bacteria showed typical rod shaped or vibroid morphotypes (1 µm x 3-5 µm).

The amount of bacteria hybridizing with probe PLA46, which is specific for *Planctomycetales,* comprising the genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera,* was also subjected to seasonal variations. The *Planctomycetales* were abundant in spring, summer, and autumn river snow, with specific cell counts of 9 ± 5%, 11 ± 6%, and 10 ± 2%, respectively, but declined to 2 ± 2% of the total cell counts in the winter river snow aggregates. Statistical analysis of specific cell counts revealed that the *Planctomycetales* subpopulation in winter river snow was significantly different from all other seasons (n = 10, $p = 0.0069$ between winter and spring, $p = 0.0036$ between winter and summer, $p = 0.0183$ between winter and autumn). Probe PLA46 mainly hybridized with budding bacteria, occurring in rosette shaped microcolonies (Fig. 3 E and F). These microcolonies consisted of large numbers of spherical cells, typically linked at the distal tips by stalks, which is a characteristic feature of *Planctomycetales* (Staley 1968, Staley et al. 1992). A smaller amount of bacteria hybridizing with probe PLA46 occurred as single cells attached to inorganic particles.

Gram-positive bacteria with a high GC content of DNA, *Legionellaceae* and *Bacteroides* occurred only in low numbers as single cells after specific hybridization with the respective probes HGC69a, LEG705 and BAC303. Methanogenic bacteria (probes MSMX860 and ARCH915) and clostridia (probe CLOBU1022) could not be detected in situ within any river snow sample.

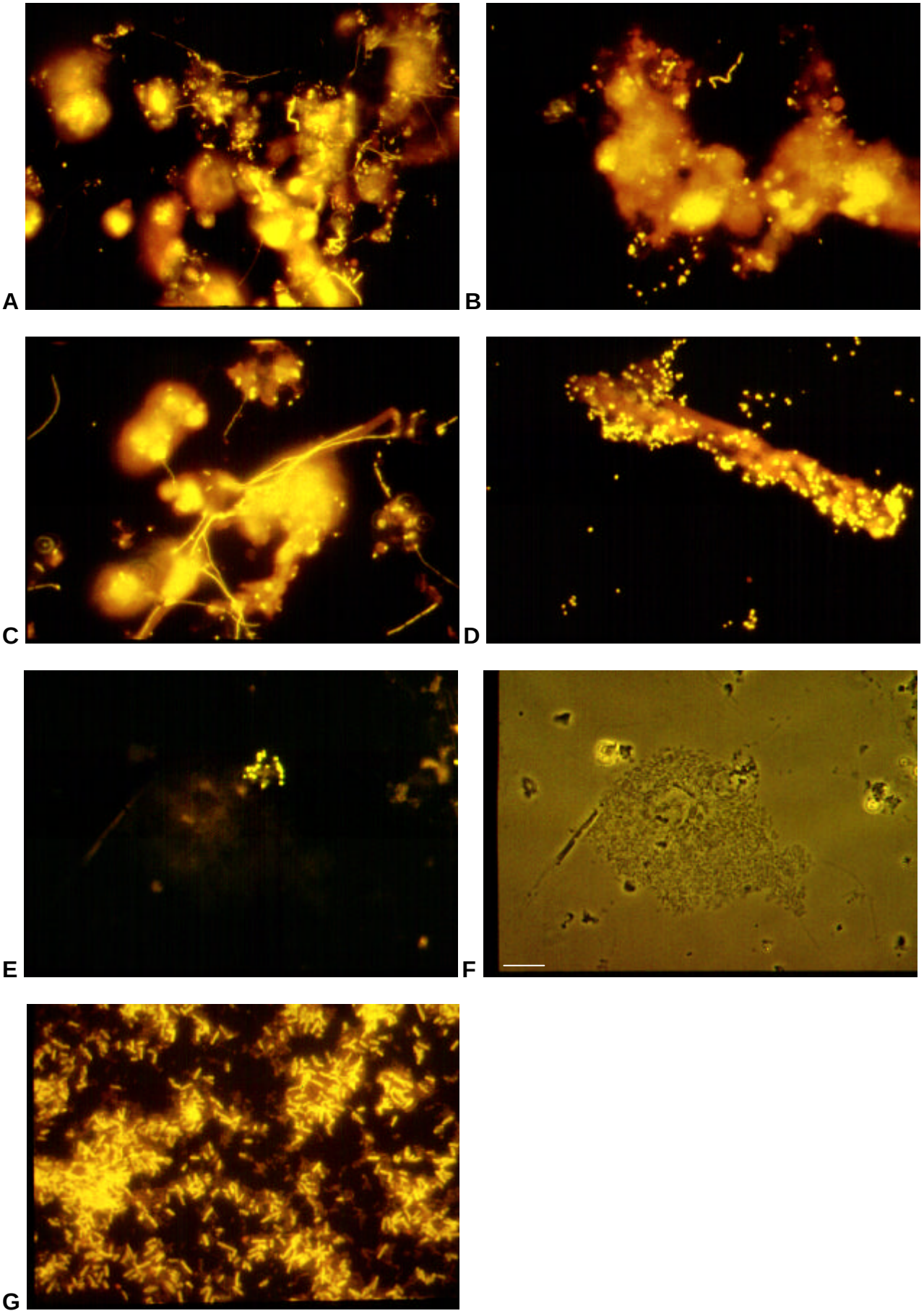


Fig. 3A-G

Fig. 3. Phase contrast and epifluorescence photomicrographs of Elbe river snow and Elbe river snow enrichment culture obtained in spring and summer. **A**: river snow obtained in spring after specific hybridization with probe EUB338. **B**: river snow obtained in summer after specific hybridization with probe beta8b. **C**: river snow obtained in spring after specific hybridization with probe CF319a/b. **D**: an anaerobically incubated methanol enrichment culture of summer river snow after specific hybridization with probe MSMX860. **E**: phase contrast micrograph of summer river snow. **F**: corresponding micrograph after specific hybridization with probe PLA46. **G**: an anaerobically incubated glucose enrichment culture of summer river snow after specific hybridization with the newly developed probe CLOBU1022. All micrographs were done at 1000 x magnification, scale bar given in F equals 10 µm and is representative for all micrographs.

3.3. *Cultivation, isolation and identification of aerobic river snow bacteria*

A comparative overview of the obtained river snow isolates, the media used for cultivation, and the results of their phenotypical and phylogenetic characterization is given in Table 5. After 2 days of incubation, bacteria growing on R2A-agar formed white, beige, yellow, orange and violet coloured colonies of sizes ranging between 2 and 5 mm. Colonies of metallic brightness on MacConkey-agar occurred after 2 to 7 days, whereas pin-point colonies of white or beige colour occurred after 2 to 4 weeks on freshwater basal medium. The predominant cell morphologies were motile, rod-shaped cells of different sizes. In total, 26 different aerobic bacterial strains could be isolated from the river snow samples. The strains were pre-characterized by Gram-staining, the determination of oxidase- and catalase-activity, their growth ability on Endo-agar, and the oxidative and/or fermentative degradation of glucose. The majority of the aerobic river snow isolates showed a Gram-negative cell wall structure. Isolates affiliated to the *Aeromonadaceae*, *Pseudomonadaceae* and flavobacteria were characterized with the API-NE profile confirming the results of the 16S rDNA derived phylogeny as shown in Table 5. Only isolates belonging to the *Enterobacteriaceae* and *Klebsiella sp*. could be identified with the API-E profile. Due to their insufficient utilization of the offered API-substrates, none of the isolates affiliated to the alpha- and beta-*Proteobacteria* could be identified using the API-profile.

In contrast to their in situ presence no members of the *Planctomycetales* could be isolated from the river snow community not even by the application of a comprehensive suite of different solid and liquid media.

TABLE 5. Phenotypical, genotypical and phylogenetic characterization of Elbe river snow isolates

Elbe river snow isolate, GenBank accession numbers	Medium used for isolation	Phenotypical identification by API-profile	Phylogenetical affiliation, closest relative as shown in Fig. 4
strain 26, AF150703	R2A-agar	none	alpha-*Proteobacterium, Rhizobium sp.67*
strain 28, AF150704	FBM[b] plus glucose and peptone	none	alpha-*Proteobacterium, unidentified bacterium 183*
strain 39, AF150709	PYGV-agar	none	alpha-*Proteobacterium, Sinorhizobium sp.*
strain 40, AF150710	PYGV-agar	none	alpha-*Proteobacterium, Azospirillum species 6*
strain P5, AF150720	PYGV-agar	none	alpha-*Proteobacterium, Sinorhizobium fredii*
strain 11, AF150689	FBM[b] plus glucose	none	beta-*Proteobacterium, Hydrogenophaga palleronii*
strain 14, AF150694	FBM[b] plus glucose	none	beta-*Proteobacterium, Alcaligenes sp.* NKNTAU
strain 18, AF150698	FBM[b] plus glucose	none	beta-*Proteobacterium, Iodobacter fluviatile*
strain 21, AF150702	FBM[b] plus glucose	none	beta-*Proteobacterium, Matsuebacter chitosanotabidus*
strain 34, AF150707	FBM[b] plus glucose	none	*beta-Proteobacterium, unidentified bacterium 348*
strain P10, AF150719	FBM[b] plus glucose	none	*beta-Proteobacterium, strain 34*
strain 4, strain 23,	R2A-agar	*Aeromonadaceae*	*gamma-Proteobacterium, Aeromonas species*
strain 10, AF150688	R2A-agar	*Aeromonadaceae*	
	R2A-agar	*Aeromonadaceae*	
strain 7, AF150712	R2A-agar	*Aeromonas hydrophila*	*gamma-Proteobacterium, Aeromonas hydrophila*
strain 19, AF150699	R2A-agar	*Aeromonas sobria*	*gamma-Proteobacterium, Aeromonas veronii*
strain 6, AF150711,	R2A-agar	*Pseudomonas alcaligenes*	*gamma-Proteobacterium,*
strain 15	R2A-agar		*Pseudomonas pseudoalcaligenes*
strain 2, AF150701	R2A-agar	*Pseudomonas putida*	*gamma-Proteobacterium, Pseudomonas putida*
strain E1, AF150717	R2A-agar	none	*gamma-Proteobacterium, Pseudomonas mandelii*
strain E3, AF150718	R2A-agar	none	*gamma-Proteobacterium, Pseudomonas sp.*
strain 1a, AF150700	MacConkey-agar	*Enterobacter cloacae*	*gamma-Proteobacterium, Klebsiella pneumonia*
strain 12, AF150693	MacConkey-agar	*Klebsiella planticola*	*gamma-Proteobacterium, Klebsiella sp.*
strain PS1, AF150721,	AMM[a] plus pyruvate and sulfate	none	*gamma-Proteobacterium, strain 1a*
strain PS2		none	
strain 32, AF150691	FBM[b] plus glucose	none	*gamma-Proteobacterium, Acinetobacter johnsonii*
strain 8, AF150713	R2A-agar plus casitone	*Flavobacteria*	Bacteroides and *Cytophaga-Flavobacteria* group, *Flavobacterium columnare*
strain 9.3, AF150715,	AMM[a] plus glucose or cellulose	none	Bacteroides, *Bacteroides ovatus*
strain 14.5		none	
strain 3, AF150705	MacConkey-agar	none	Firmicutes with a low GC content of DNA, *Staphylococcus warneri*
strain 9.1, AF150714	AMM[a]plus glucose	none	Firmicutes with a low GC content of DNA, *Clostridium butyricum*
strain 11.1, AF150690	AMM[a] plus casamino acids	none	Firmicutes with a low GC content of DNA, *Clostridium glycolinum*
strain 11.2, AF152959	AMM[a] plus casamino acids	none	Firmicutes with a low GC content of DNA, *Clostridium propionicum*
strain 11.3, AF150692	AMM[a] plus casamino acids	none	Firmicutes with a low GC content of DNA, *Clostridium bifermentans*
strain 15.4, AF150696	AMM[a] plus chitin	none	Firmicutes with a low GC content of DNA, *Clostridium quinii*
strain 15.3, AF150695	AMM[a] plus chitin	none	Firmicutes with a low GC content of DNA, *Holdemania filiformis*
strain 14.3,	AMM[a] plus cellulose or chitin	none	Firmicutes with a low GC content of DNA,
strain 15.5, AF150697		none	*Anaerofilum agile*
strain C2, AF150716	FBM[b] plus glucose and peptone	none	Firmicutes with a high GC content of DNA, *Arthrobacter oxidans*
strain 37, AF150708	FBM[b] plus glucose and peptone	none	Firmicutes with a high GC content of DNA, *Mycobacterium sp.28*
strain PS5, AF150722,	AMM[a] plus pyruvate and sulfate	none	Firmicutes with atypical cell wall,
strain PS6		none	*Anaeromusa acidoaminophila*
strain SRB3, AF159535	AMM[a] plus pyruvate or butyrate or acetate and sulfate	none	delta-*Proteobacterium,*
strain SRB4, AF150723		none	*Desulfovibrio desulfuricans*
strain SRB7, AF159536		none	
strain SRB19, AF159534		none	
MS-enriched culture	AMM[a] plus formate	*Methanosarcinaceae* [c]	*Archaea*

[a] AMM: anaerobic mineral medium (Tschech & Pfennig 1984); [b] FBM: freshwater basal medium;
[c] identified by FISH and cell morphology

3.4. *Cultivation, isolation and identification of anaerobic river snow bacteria*

In total 14 different anaerobic strains could be isolated from the river snow aggregates (Table 5). They were grouped according to their utilization of carbon sources, and the use of electron donors and electron acceptors.

Sulfate reducing bacteria were identified within 5 to 21 days after inoculation by in situ hybridization with probe SRB385Db. After several passages in deep agar dilutions, four SRB strains (strains SRB3, SRB4, SRB7 and SRB19), displaying different colony- and cell-morphologies could be isolated. Phylogenetic analysis, however, revealed all of them as close relatives to *Desulfovibrio desulfuricans* (Table 5). Growth of methanogens was detected after 14 to 21 days of incubation in acetate, methanol and formate enrichment cultures. The typical globular shape of the microcolonies indicated them as members of the *Methanosarcinaceae*, confirmed by specific in situ hybridization with probes ARCH915 and MSMX860 (Fig. 3D). From the anaerobic chitin enrichment culture, a bacterium displaying a striking morphology of long, thin, unregular curved filaments could be isolated (strain 15.3) that is related to *Holdemania filiformis.* Two further anaerobic isolates (PS5 and PS6) grew on pyruvate supplemented medium and were closely related to *Anaeromusa acidoaminophila.*

In addition to the obligate anaerobic bacteria, several clostridia could be successfully isolated from the river snow community. Six different bacterial isolates (strains 9.1, 11.1, 11.2, 11.3, 15.4, 14.3), which were characterized by their thick, rod shaped cells and the occurrence of endospores, could be obtained using glucose, casamino acids or chitin as sole carbon source. In situ hybridization with the newly developed probe CLOBU1022, specific for strains 9.1, 9.2, 9.4 and *Clostridium butyricum*, did not show fluorescent cells in the Elbe river snow sample. However, strong hybridization signals of typically rod shaped bacteria, which contained

endospores, could be detected in river snow enrichment cultures incubated under anaerobic conditions (Fig. 3G).

3.5. *Phylogenetic analysis of the isolated river snow bacteria*

The phylogenetic trees given in Fig. 4 A-I reflect the phylogenetic relationships of river snow isolates to their next known relatives. The accession numbers of the river snow isolates deposited in GenBank are given in Table 5.

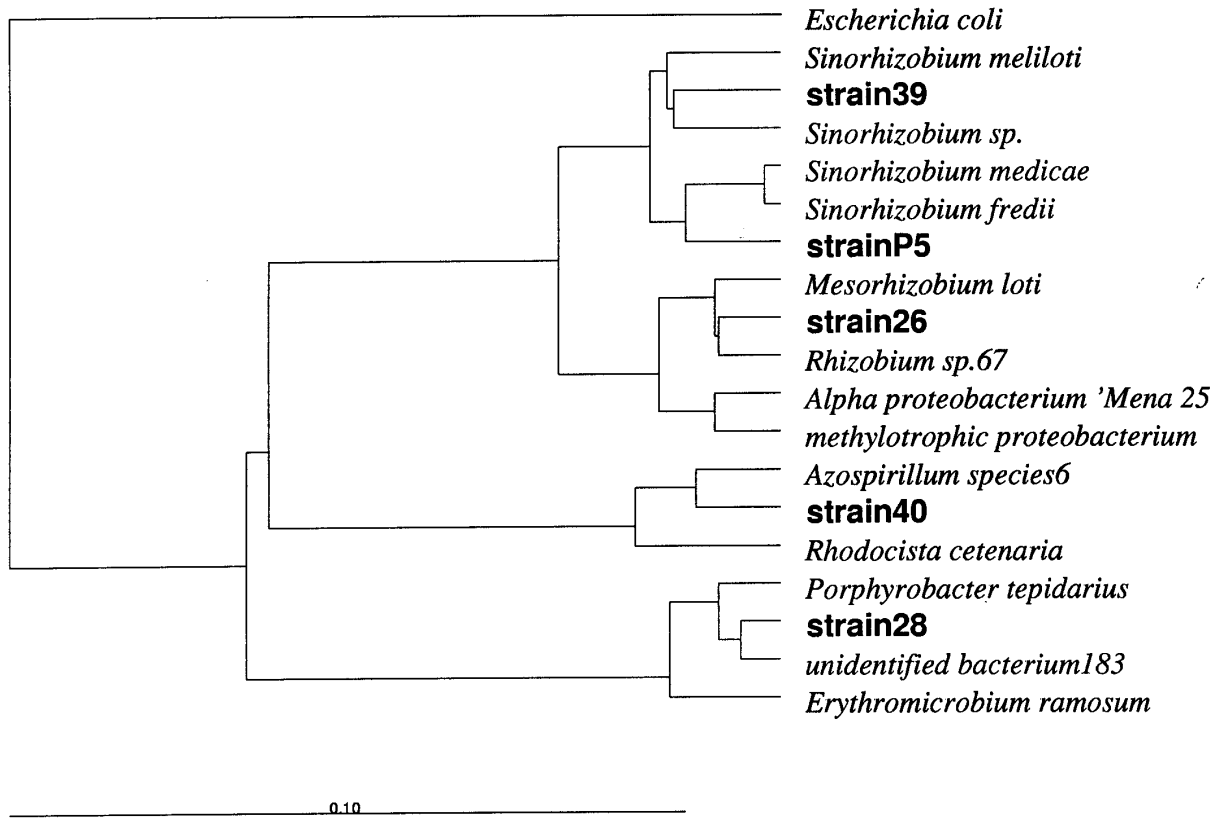


FIG. 4A. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of the 5 bacterial river snow isolates affiliated to the alpha-*Proteobacteria* to their closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

The five bacterial river snow isolates affiliated to the alpha-*Proteobacteria,* were equally distributed among this lineage as shown in Fig. 4A. Next known relative to strain 39 was *Sinorhizobium sp*. with a 16S rDNA sequence identity of 97%. Strain P5 was also closely related to the *Sinorhizobium* group with 98.1% sequence identity with *Sinorhizobium fredii* and 96.1% sequence identity with strain 39. *Rhizobium sp.67* was the next known relative to strain 26 with 97.4% sequence identity and *Azospirillum species 6* was closely related to strain 40 with 97.5% sequence identity. Whereas all these relatives are wellknown bacteria ubiquitous in soil and aquatic habitats, the closest relative to strain 28 with a sequence identity of 98.3% was an unidentified bacterium 183 isolated from crop field soil in New Zealand.

Six Elbe river snow isolates were affiliated to the beta-subclass of *Proteobacteria* (Fig. 4B). Three of them (strains 11, 34, and P10) formed a distinct cluster among the beta1-subclass of *Proteobacteria.* Strain 34 was closely related to the unidentified bacterium 348 (sequence identity 98.2%). This bacterium belonged to the bacterial community of activated sludge of a large municipal wastewater treatment plant investigated by Snaidr et al. (1997). Strain P10 was closely related to strain 34 with 95.8% sequence identity. *Hydrogenophaga palleronii* with the ability to grow aerobically with 4-aminobenzenesulfonate described by Blumel et al. (1998) was the next relative to strain 11 with sequence identity of 96.8%. Strain 21, also affiliated to the beta1-subclass of *Proteobacteria* was closest related (sequence identity 99.1%) to *Matsuebacter chitosanotabidus.* This bacterial strain was isolated from soil collected in Matsue, Japan, and produces a novel, previously unreported chitosanase (Park et al.1999). With a sequence identity of 95.3% strain 21 was furthermore related to *Aquabacterium commune* (beta *Proteobacterium* B8), an in situ dominant bacterial species from the Berlin drinking water system (Kalmbach et al. 1999). In contrast to these Elbe river snow isolates, strains 14 and 18, were affiliated to the beta2-subclass of *Proteobacteria*. Strain 14 was closest related to *Alcaligenes sp.*

NKNTAU, a nitrate-reducing *Alcaligenes sp*. capable of utilizing taurine (Denger et al. 1997) with a sequence identity of 99.2%. Next known relative to strain 18 was *Jodobacter fluviatile*, a *Chromobacterium* and typical inhabitant of riverine systems (Sneath 1984) with a sequence identity of 89.8%.

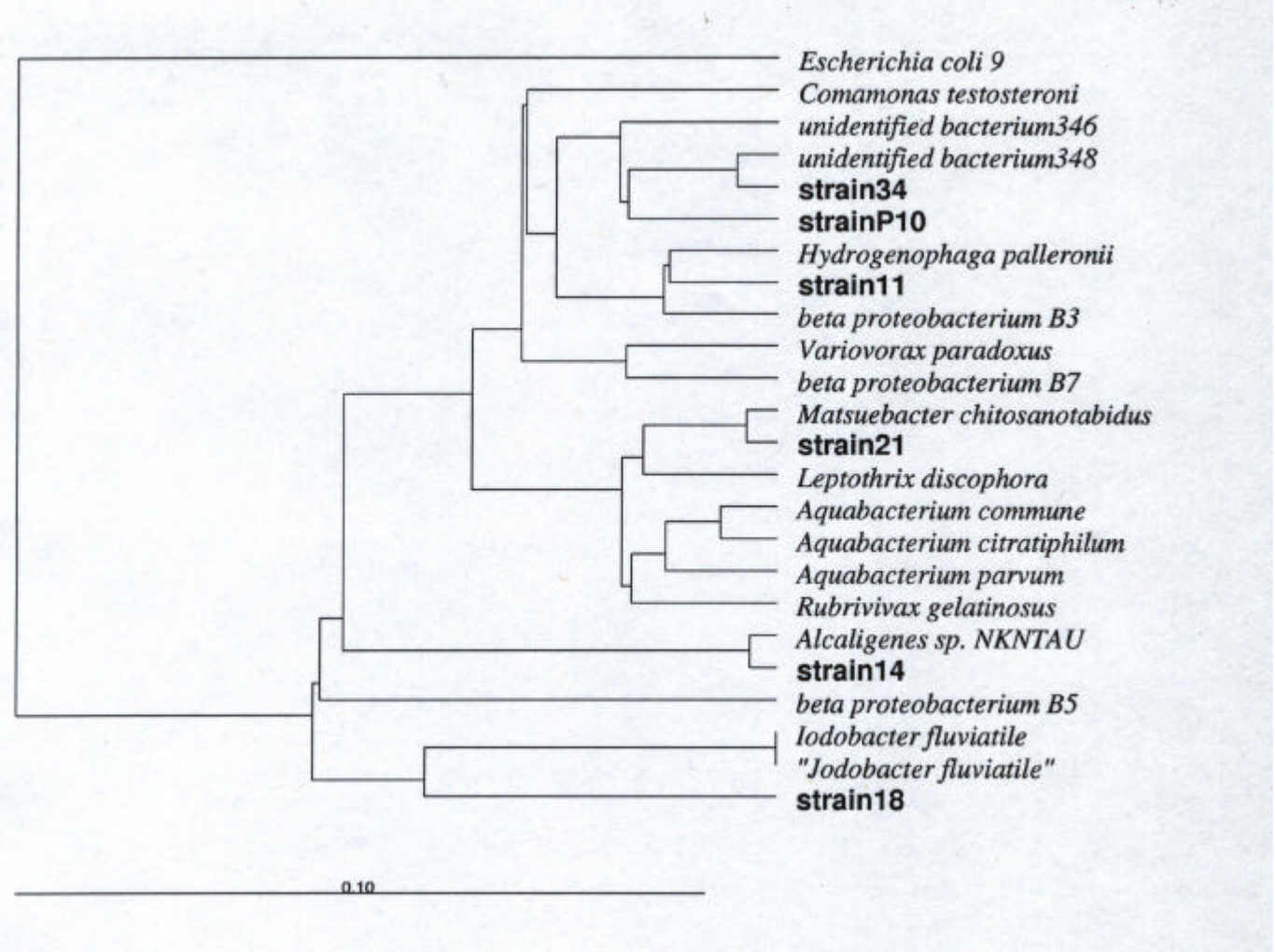


FIG. 4B. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of the 6 bacterial river snow isolates affiliated to the beta-*Proteobacteria* to their closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

The majority of the aerobic river snow isolates, 11 different strains, were members of the gamma-*Proteobacteria* (Fig. 4C) and equally distributed among this lineage. Three of them were affiliated to the *Klebsiella* group strain 12, PS1 and 1a. With a sequence identity of 99.8% strain 12 was nearly identical with *Klebsiella sp*. and also

closely related to strain PS1 (0.7% sequence difference). Next known relative to strain 1a was *Klebsiella pneumonia* with 98.3% sequence identity. Three further isolates, strain 7, 10 and 19 were affiliated to the *Aeromonadaceae.* All of them were closely related to their next known bacterial relatives with 16S rDNA sequence identities ranging from 99.1% to 99.4%. Closest relative to strain 7 was *Aeromonas caviae*, a fish pathogen and *Aeromonas species* to strain 10, respectively. Both were closely related to *Aeromonas hydrophila,* the type strain of the diverse group of motile aeromonads, capable of causing disease in fish, frogs and several other animals (Farmer et al. 1992). Closest relative to strain 19 was *Aeromonas veronii*, defined by Hickmann-Brenner et al. (1987) as Aeromonas strains, that are ornithine-decarboxylase positive and biochemically very similar to *Vibrio cholerae*. 16S rDNA sequence analysis revealed strain 6, 2, E3 and E1 as members of the family *Pseudomonadaceae*. Sequence identities ranged from 99.8% to 98.7%. Closest relative to strain 6 was *Pseudomonas pseudoalcaligenes* and strain 2 was closely related to *Pseudomonas putida*. Strain E3 had highest sequence identity with *Pseudomonas sp*., whereas strain E1 next known relative was *Pseudomonas mandelii*. Strain 32 was affiliated to the *Acinetobacter* group with *Acinetobacter johnsonii* as closest relative with a sequence identity of 98.2%.

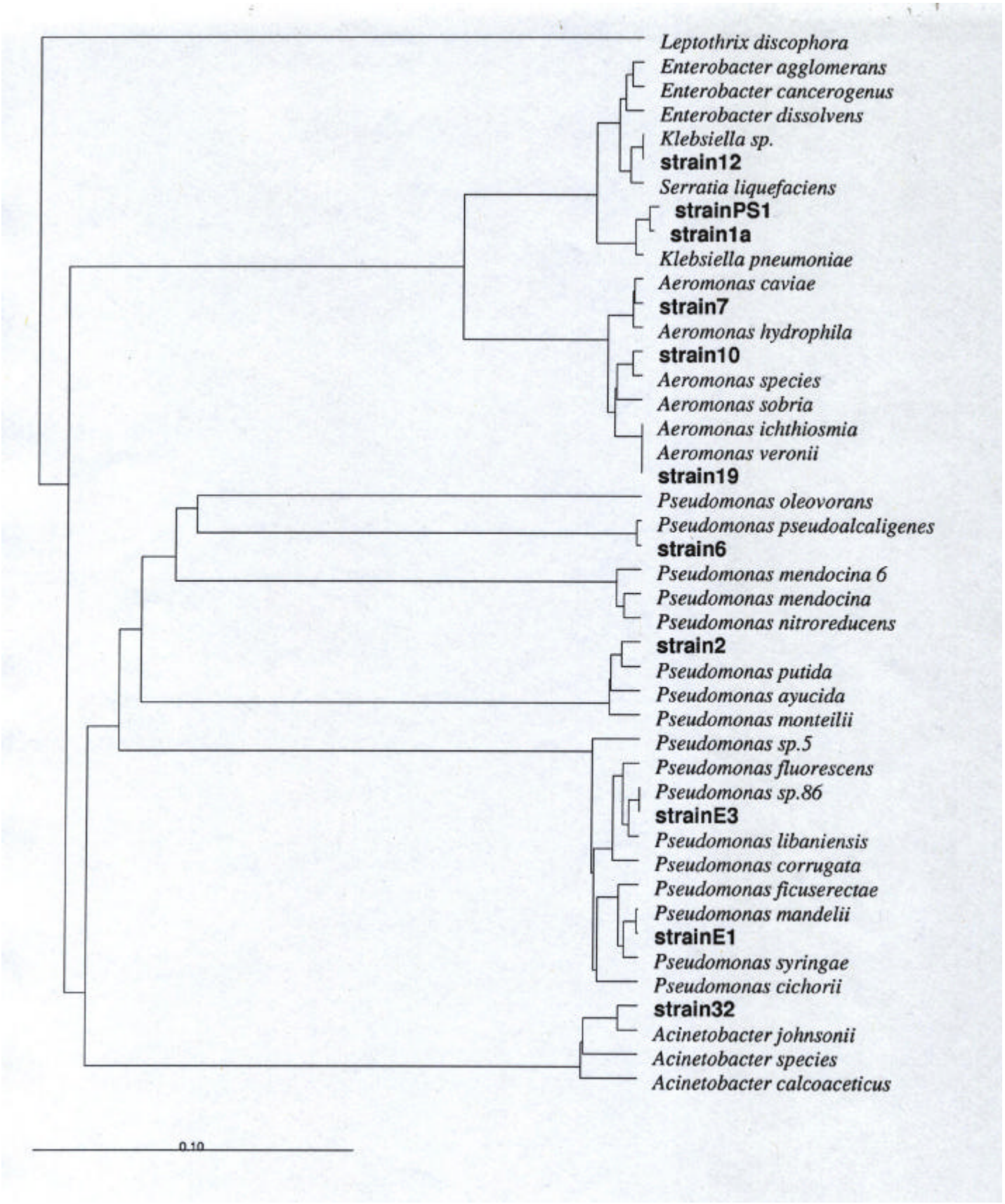


FIG. 4C. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of the 11 bacterial river snow isolates affiliated to the gamma-*Proteobacteria* to their closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

Two members of the Elbe river snow isolates, strain 8 and strain 9.3, were identified as members of the *Cytophaga Flavobacteria Bacteroides* (CFB) group shown in Fig. 4D. Closest relative to strain 8 was *Flavobacterium columnare* with a sequence identity of 97.3%. A predominance of Flavobacteria in river and lake samples has been reported in several studies (for example, Flint 1989, Kenzaka et al. 1998, Sugita et al. 1982). Strain 9.3 was closely related to *Bacteroides eggerthii* and *Bacteroides ovatus* which could be isolated from the colon of humans and animals.

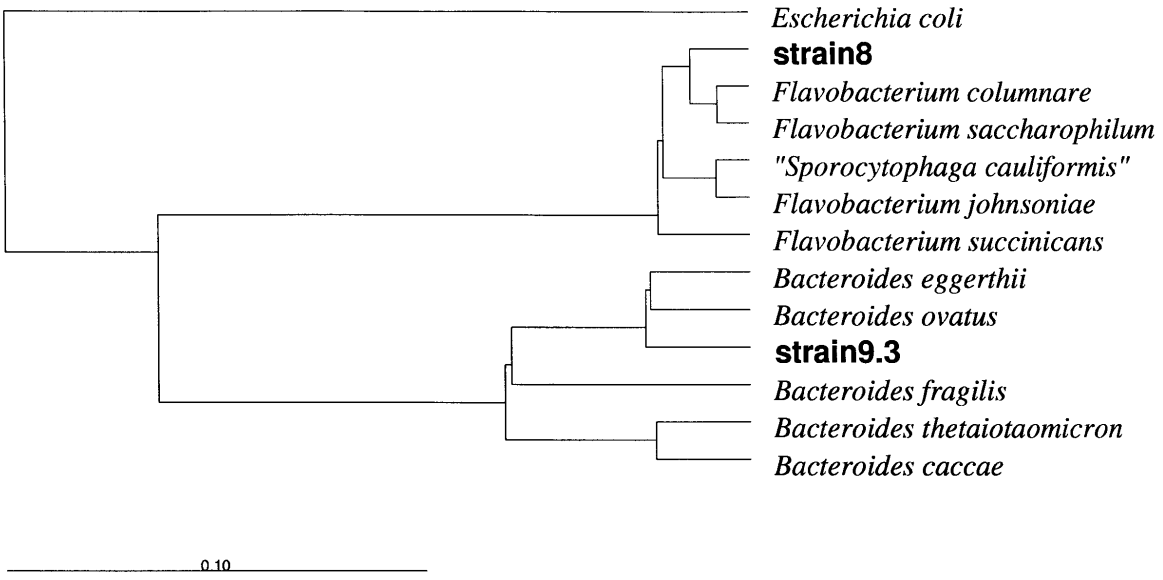


FIG. 4D. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of the 2 bacterial river snow isolates affiliated to the *Cytophaga-Flavobacteria Bacteroides* (CFB) group to their closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

From the isolated river snow bacteria, which were affiliated to firmicute bacteria with low GC content of DNA, only strain 3 was closely related to members of the genus *Staphylococcus* (Fig. 4E). Next known relative to strain 3 was *Staphylococcus warneri* with a sequence identity of 99.1%. Staphylococci are widespread in nature, though they are found more consistently and in denser populations on the skin, skin glands, and mucous membranes of mammals and birds (Kloos et al. 1992).

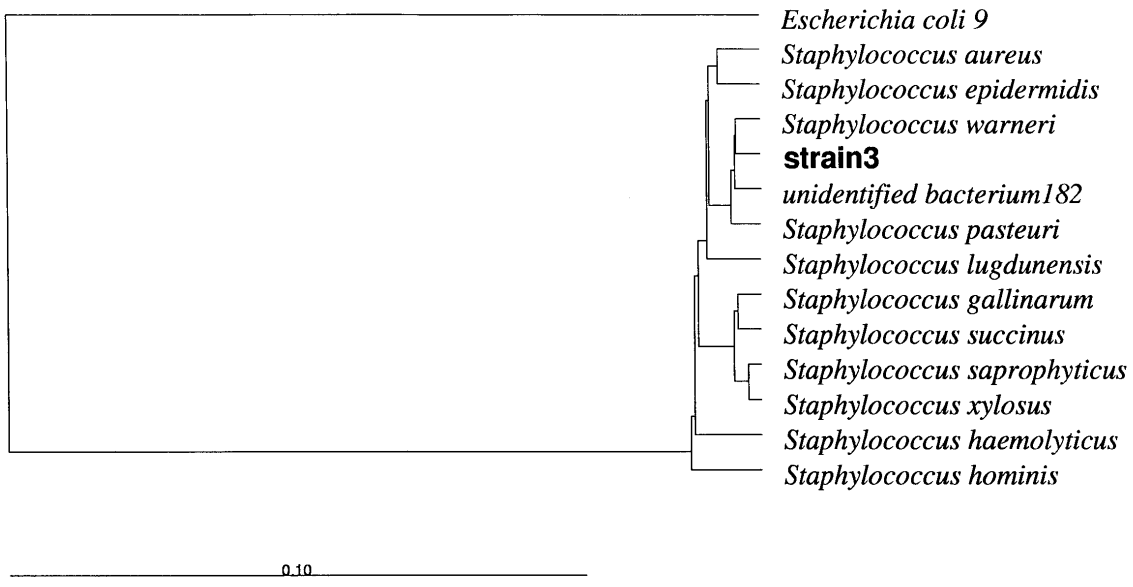


FIG. 4E. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of strain 3 affiliated to the genus *Staphylococcus* to its closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

From anaerobic cultivation the majority of the bacterial isolates were affiliated to the firmicute bacteria with low GC content of DNA. They were members of the clostridia-branch shown in Fig. 4F, with different sequence identities to their next relatives ranging from 99.7% to 96.5%. The genus *Clostridium* includes psychrophilic, mesophilic, and thermophilic species. Most of the clostridial species stain Gram-positive and are motile with peritrichous flagellation. The major role of these organisms in nature is the fermentative degradation of organic material to acids, alcohols, CO_2, H_2, and minerals (Hippe et al. 1992). Next known relative to strain 11.3 was *Clostridium bifermentans*, wheras strain 11.1 was closely related to *Clostridium glycolinum*. Strain 9.1 had closest sequence identity with *Clostridium butyricum*, whereas strain 15.4 was related to *Clostridium quinii* and strain 11.2 to *Clostridium propionicum*. Strain 15.3 was only distantly related to its next relative *Holdemania filiformis,* an Eubacterium-like strain, phylogenetically a member of the *Clostridium* subphylum, showing a sequence identity of 91.1%. *Holdemania filiformis* (gen. nov., sp. nov.) contains a novel type B wall murein and was recently isolated from human feces (Willems et al. 1997). Strain 15.5 was closely related to *Anaerofilum agile* with a sequence identity of 98.6%. *Anaerofilum agile* is a strictly anaerobic, mesophilic, acidogenic bacterium isolated from the biofilm population of a fixed-film treating sour whey (Zellner et al. 1996).

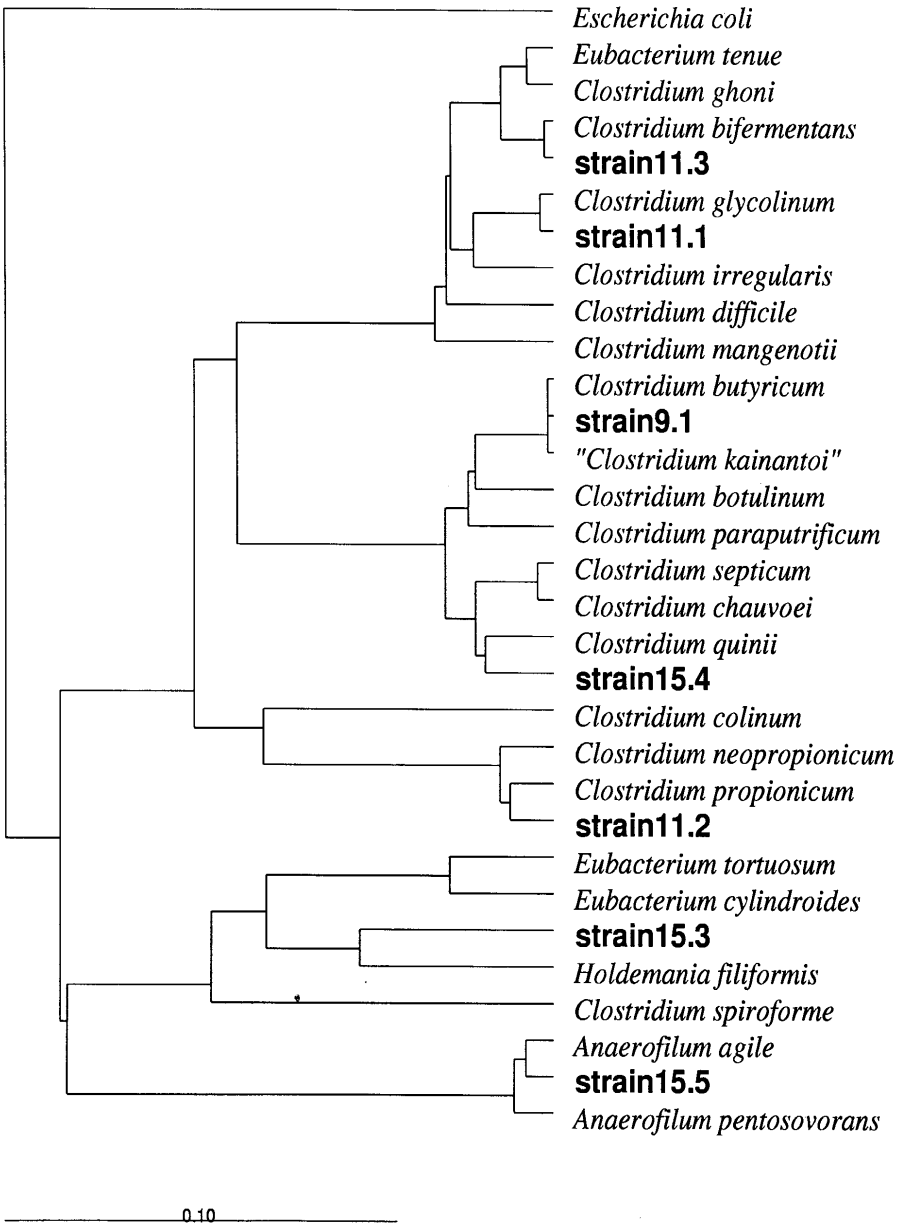


FIG. 4F. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of the 7 bacterial river snow isolates affiliated to the firmicute bacteria with low GC content of DNA, the clostridia-branch, to their closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

Two bacterial isolates from the Elbe river snow community, strain 37 and strain C2, were affiliated to firmicute bacteria with high GC content of DNA (Fig.4G). Next known relative to strain 37 was *Mycobacterium* sp. 28 with a sequence identity of 98%. *Mycobacterium* sp. 28 was isolated from fossil-fuel-contaminated environments and is capable of degrading polycyclic aromatic hydrocarbons (Gowindaswami et al. 1995). Strain C2 was closely related to *Arthrobacter oxydans* with a sequence identity of 98.5%. Members of the genus *Arthrobacte*r are typical soil inhabitants but were also isolated from sewage and activated sludge (Jones & Keddie 1992).

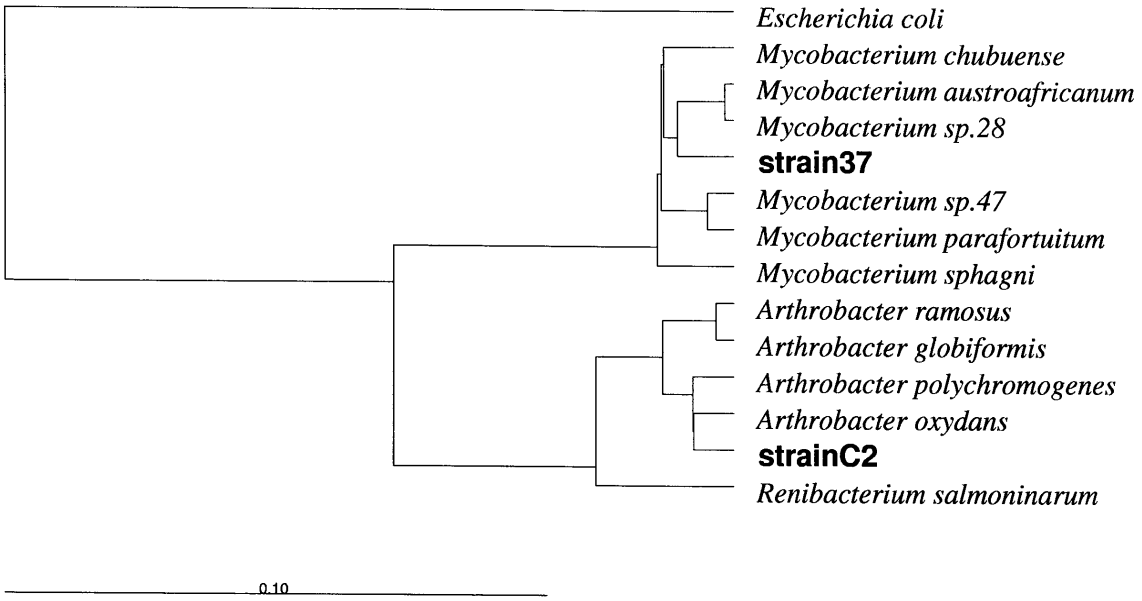


FIG. 4G. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of the 2 bacterial river snow isolates affiliated to the firmicute bacteria with high GC content of DNA to their closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

As demonstrated in Fig 4H, a single bacterial isolate, strain PS5, belonged to firmicute bacteria with atypical cell wall, to the genera Sporomusa. These anaerobic spore-formers posses a Gram-negative type of cell wall. Strain PS5 was related to *Anaeromusa acidaminophila* (gen. nov., comb. nov.) with a sequence identity of 96.9%. This thermophile bacterium formed an individual line of descent and was equidistantly placed between *Dethiosulfovibrio peptidovorans* and *Anaerobaculum thermoterrentum*, (similarity of 85%), both of which also form single lines of descent (Baena et al. 1999).

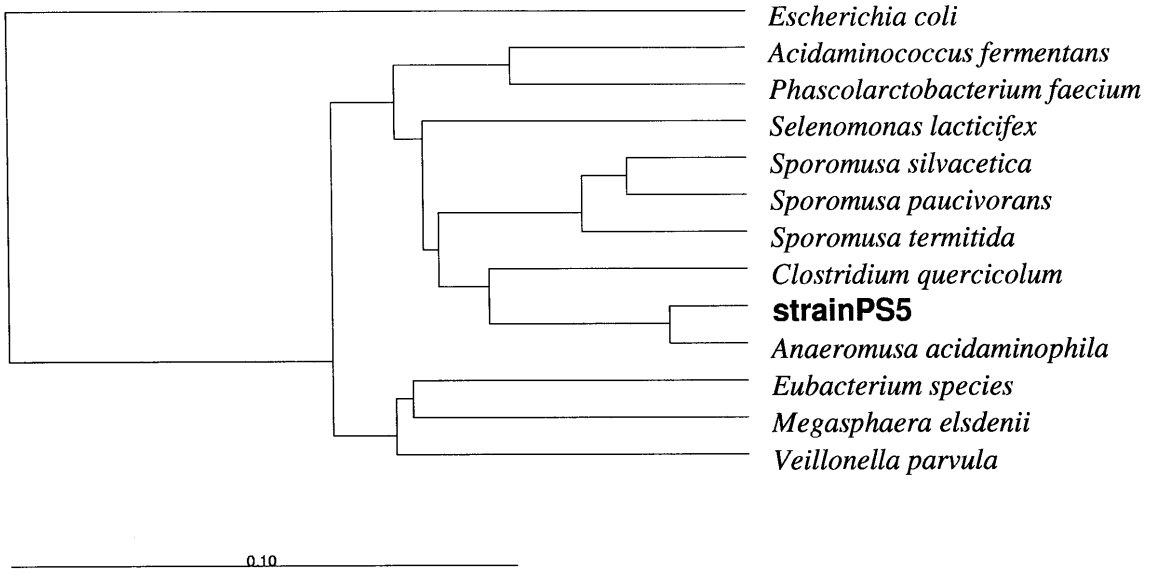


FIG. 4H. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of strain PS5 affiliated to the firmicute bacteria with atypical cell wall to its closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

Sequence analysis of the four bacterial isolates affiliated to the delta-*Proteobacteria,* the sulfate-reducing-bacteria revealed all of them as identical to each other and closest related to *Desulfovibrio desulfuricans* with a sequence identity of 99.2%.

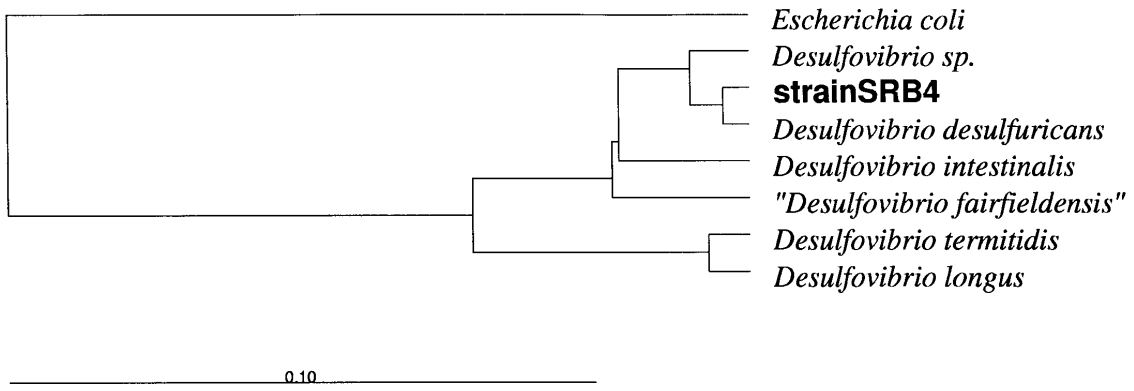
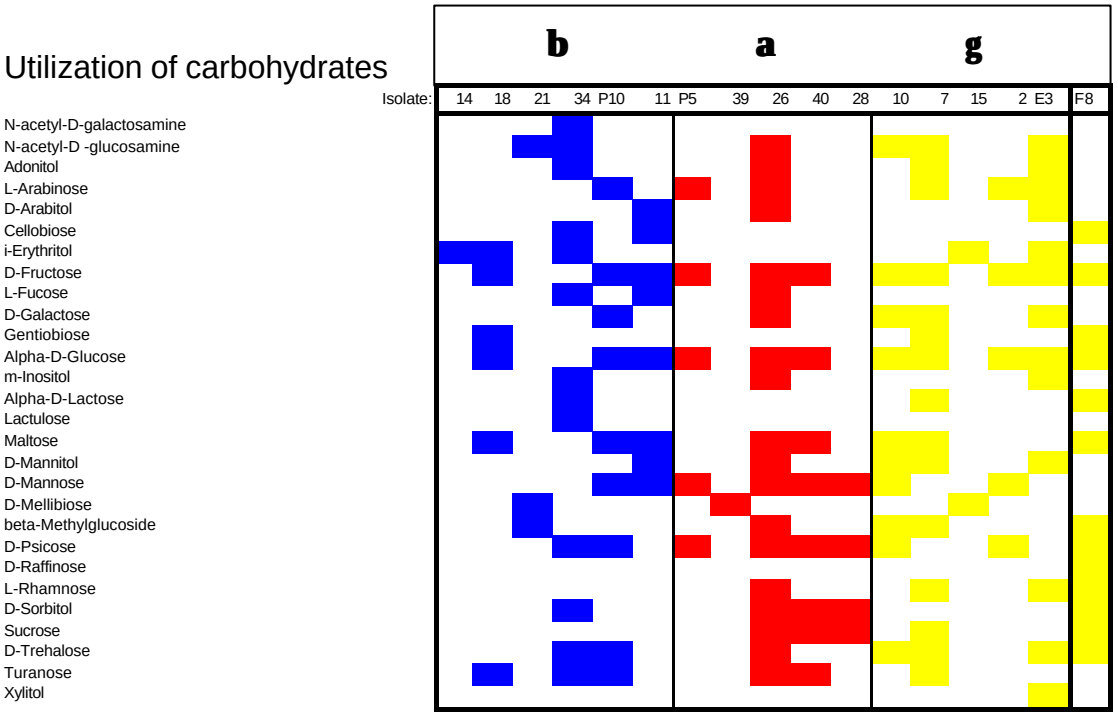
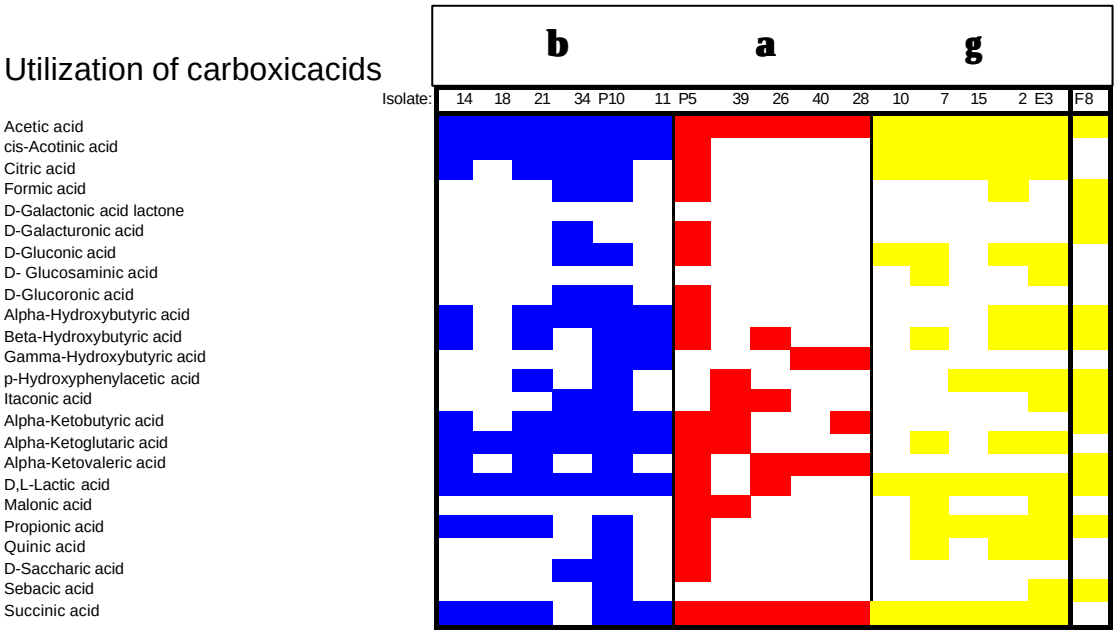


FIG. 4I. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of strain SRB4 affiliated to the delta-*Proteobacteria*, the sulfate-reducing-bacteria to its closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

The physiological potential of 16 isolated aerobic Elbe river snow bacteria, and strain F8 is demonstrated in Table 6. Although the test was performed with all isolated bacteria, a variety of them completely failed to grow in the test system or gave false positive results in all 96 test wells.

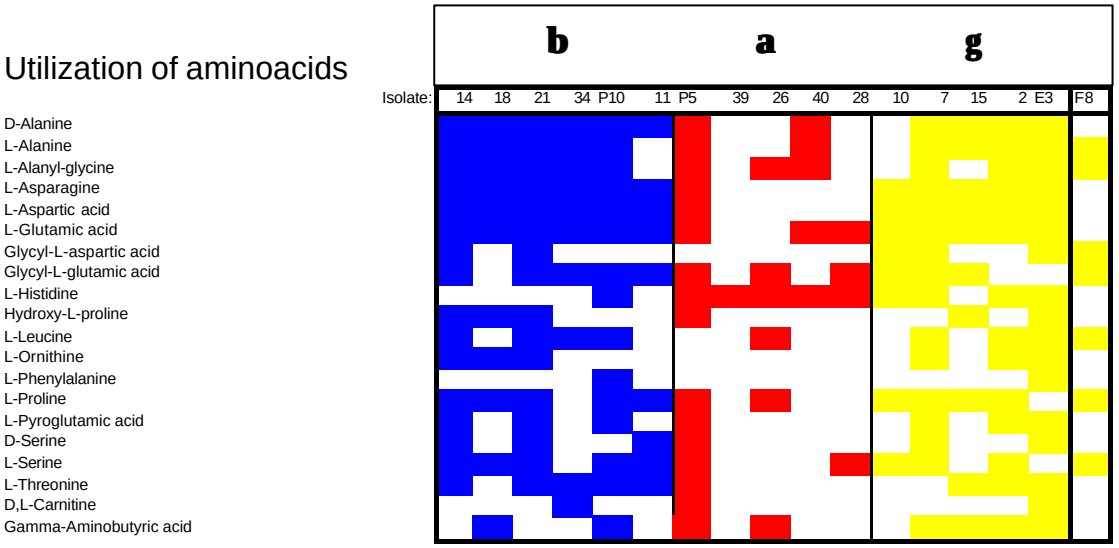
TABLE 6 BIOLOG data of all Elbe river snow isolates affiliated to the beta-*Proteobacteria*, alpha-*Proteobacteria,* a collection of isolates affiliated to the gamma-*Proteobacteria* and the South Saskatchewan river snow isolate F8.



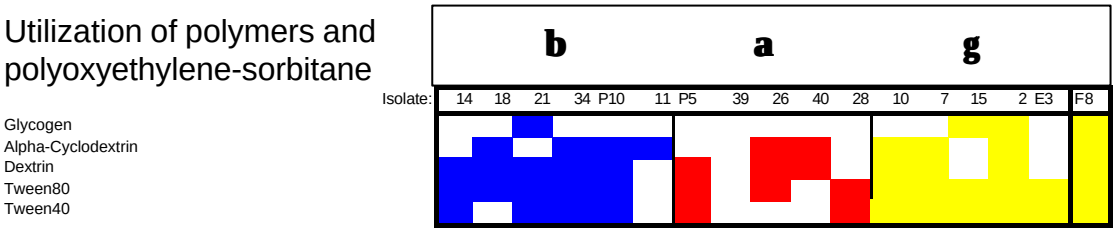


Beta-*Proteobacteria* are marked blue, alpha-*Proteobacteria* are marked red, gamma-*Proteobacteria* including strain F8 are marked yellow.

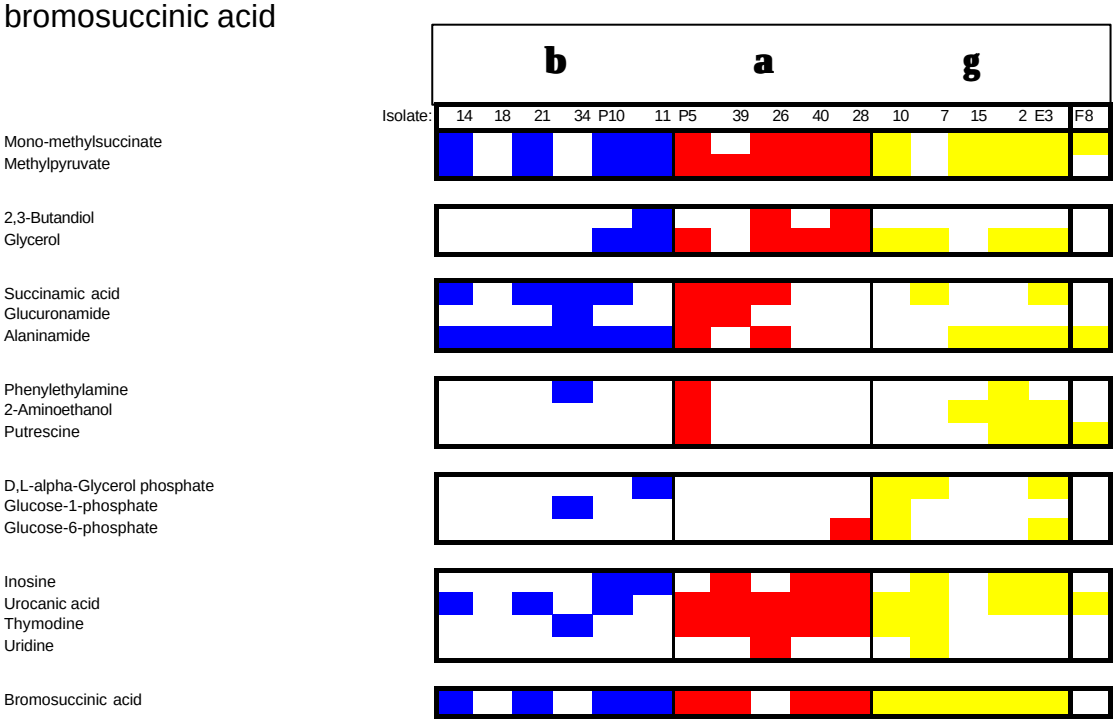
Utilization of aminoacids



Utilization of polymers and polyoxyethylene-sorbitane



Utilization of ester, alcohols, amids, amins, phosphorylated-, aromatic-,and bromosuccinic acid



Beta-*Proteobacteria* are marked blue, alpha-*Proteobacteria* are marked red, gamma-*Proteobacteria* including strain F8 are marked yellow.

Because of the low number of evaluable BIOLOG results for the isolated strains, the assignment to any subgroup seems not to imply any preferential physiological ability. Moreover in each subgroup there are strains with specific and strains with a broude range of metabolic capacities.

Nevertheless the isolated beta strains trend to focuse on aminoacids, polymers and Tween. Carbohydrates were not the preferred substrate for the beta strains. None of them was able to utilize or oxidize xylitol, sucrose, L-rhamnose und D-raffinose and only strain P10 was able to utilize D-galactose. Just four beta strains were able to degrade turanose, alpha-D-glucose and D-fructose. In contrast to the carbohydrates, most of the carboxicacids served as nutrients for the majority of the tested beta strains. Whereas acetic acid, cis-acotinic acid and D,L-lactic acid were utilized by all beta strains, none of them was able to degrade D-galactonic acid lactone, D-glucosaminic acid and malonic acid. Aminoacids were the preferred substrate for all isolated beta strains. D-alanine, L-alanine, L-alanine-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glucyl-L-glutamic acid, L-leucine, L-proline, L-serine and L-threonine were utilized by nearly all tested beta strains. Each offered aminoacid served as substrate for at least one of the strains. Solely strain 34 was able to grow on D,L-carnitine. The polymers glycogen, alpha-cyclodextrin and dextrin also served as a suitable substrate for the majority of the Elbe beta strains. Nearly all beta strains were able to utilize Tween40 (polyoxyethylenesorbitane monopalmitate) and Tween80 (polyoxyethylenesorbitane monooleate). Utilization was characterized by a rapid positive colour reaction after 1 - 2 h of incubation. Beta strain utilization of ester, alcohols, amids, amins, phosphorylated compounds, aromatic compounds and Bromosuccinic acid was indifferent with the exception of alaninamide which could be utilized by all tested beta strains.

The physiological potential of the isolated alpha strains was characterized by a minimal utilization of the offered carbon sources, with the trend focusing on the

aromatic compounds. Mono-methylsuccinate, methylpyruvate and the major part of the aromatic compounds could be utilized by nearly every isolated alpha strain. Nevertheless the alpha strains seem to represent a mixture of very versatile strains (e.g. isolate P5) and very specialized strains (e.g. isolate 26 and 39). Whereas strain P5 produced only positive colour reaction with the minority of the offered carbohydrates, it was able to utilize nearly every offered carboxylacid, aminoacid, dextrin, Tween80 and Tween40. In contrast to this, strain 26 favoured growth on the carbohydrates. The majority of the carboxicacids and aminoacids did not serve as nutrients for this strain. Strain 39 solely favoured to grow on amids, aromatic compounds and bromosuccinic acid.

The isolated strains of the gamma-*Proteobacteria* showed an indifferent physiological behaviour, but they collectively trend to focuse on the aminoacids and polymers. Whereas strain 7, strain 10 and E3 were able to utilize the majority of the offered carbohydrates, strain 2 and strain 15 could just utilize a minor part of them. The aminoacids L-asparagine, L-aspartic acid and L-glutamic acid produced positive colour reactions with all tested gamma strains, whereas hydroxy-L-proline and D,L-carnitine were solely utilized by strain 15 and strain E3. Polymers, Tween80 and Tween40 served as good nutrients for this phylogenetic group compared with the beta group. The physiological potential in degrading ester, alcohols, amids, amins, phosphorylated compounds, aromatic compounds and bromosuccinic acid was better compared with the beta strains but worse compared with the alpha group.

3.7. *In situ relevance of cultivated river snow bacteria*

Beta-*Proteobacteria* constituted up to 54% of all in situ river snow bacteria in the Elbe. Beta-*Proteobacteria* isolates were exclusively isolated either from freshwater basal medium supplemented with yeast extract and glucose, or on PYGV medium.

The importance of the beta-proteobacterial isolates as dominant members of the bacterial river snow community was demonstrated for strain 21. During all seasons, in situ probing of Elbe river snow samples with the specific probe BETA21 visualized single cells of characteristic morphology and typical inclusion bodies, accounting for at least 1% of the total cell counts (Fig. 5A). Further in situ probing of river snow obtained from the Rhine River, the Oder River, and the Spree River (Fig. 5B) also showed different amounts of this widespread beta-*Proteobacterium.* The other beta isolates (strain 11, 14, 18, 34 and P10) could not be detected with the newly developed strain specific probes in the Elbe. But hybridization with the newly developed group specific probes for strain 18 and relatives (probeClu18kl), as well as for strain 11, 34, P10 and their next relatives (probe Clu34kl and Clu34gr) was successful in other rivers. In contrast to the Elbe and Rhine River, hybridization of samples from the Oder River with probe Clu18kl resulted in the visualization of strong fluorescent cells of coccoid morphology (Fig. 5C). In young lotic microbial biofilms, cultivated in a rotating annular bioreactor fed with natural river water from the South Saskatchewan River at Saskatoon, Saskatchewan, Canada, a great number of cells of unique cell morphology could be detected with probe Clu34kl. Their amount decreased with increasing age of the biofilm. Whereas in the Elbe only a few single bacterial cells could be detected with the probes Clu34gr and Clu34kl, in the Oder and Rhine chains of fluorescent rod shaped bacteria frequently occurred (Fig. 5D).

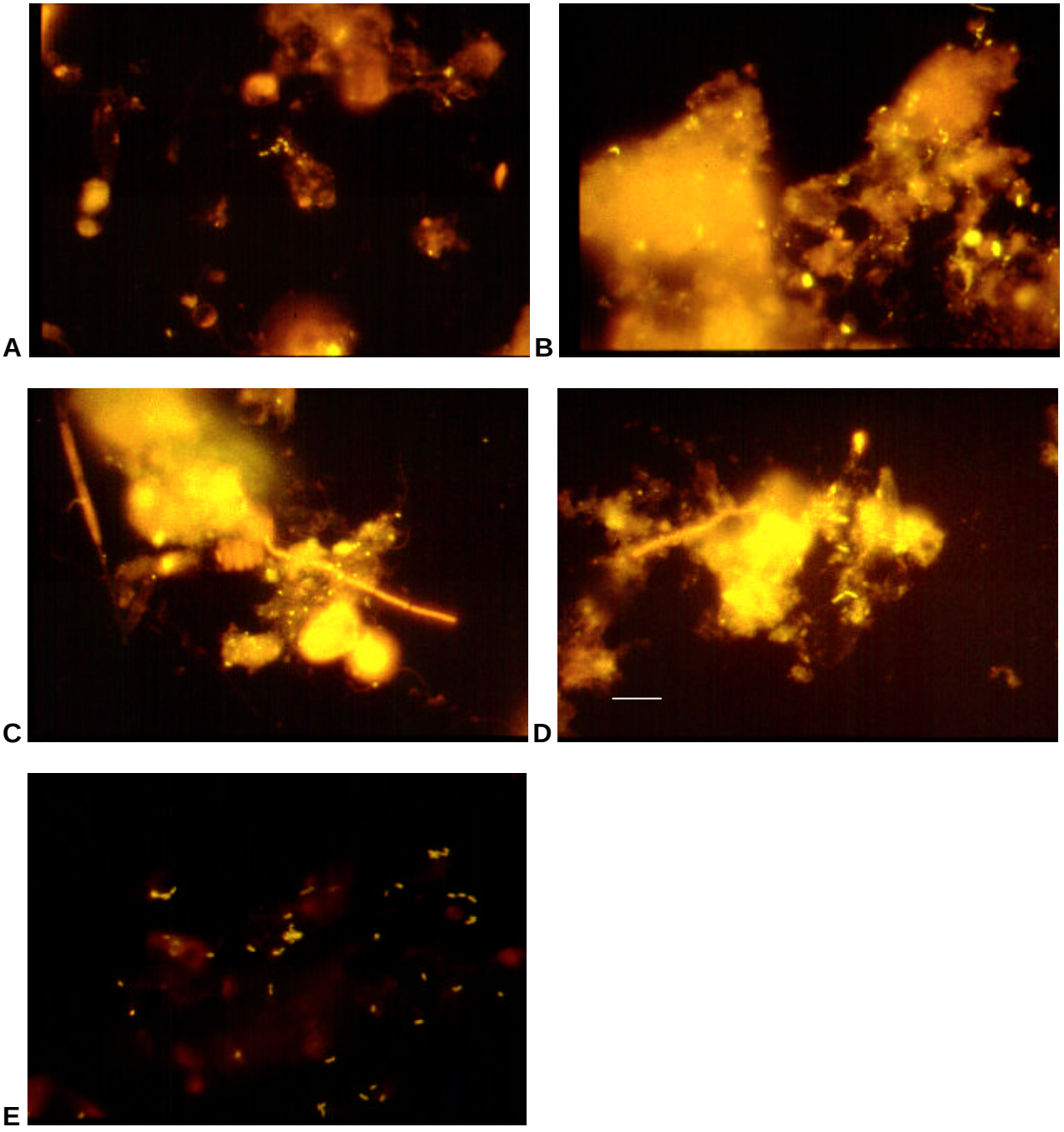


Fig. 5. Epifluorescence photomicrographs of Elbe, Oder, Rhine and Spree river snow samples obtained in spring and summer. **A**: Summer Elbe river snow sample after specific hybridization with probe BETA21. **B**: Summer Spree river snow sample after specific hybridization with probe BETA21.**C**: Summer Oder river snow sample after specific hybridization with probe Clu18kl. **D**: Summer Rhine river snow sample after specific hybridization with probe Clu34kl. **E**: Spring Elbe river snow sample after specific hybridization with probe AERO1244. All micrographs were done at 1000 x magnification, scale bar given in D equals 10 µm and is representative for all micrographs.

Cultivation on MacConkey-agar, favoured the growth of gamma-*Proteobacteria*, especially *Aeromonadaceae* and *Pseudomonadaceae* due to its amount of selective compounds (bile salts). Seasonal variations of the *Aeromonadaceae* in situ could be observed using the newly developed probe AERO1244. Although no hybridization signals could be detected in summer and autumn river snow, fairly high amounts of aeromonads reaching up to 3% of the total bacterial cell counts could be monitored in samples obtained in spring (Fig. 5E) and winter.

Although the *Cytophaga-Flavobacteria* group reached up to 36% of all bacteria in spring river snow aggregates, only one river snow isolate could be assigned to this phylum. The combined application of probes CF319a/b and FLAVO1044 proved the in situ abundance of this strain, visualizing microcolonies composed of strongly fluorescent, rod shaped bacteria (data not shown).

3.8. *Relevance of isolated beta-Proteobacteria in enrichment Elbe river snow cultures*

Elbe river snow batch cultures of sterile raw Elbe river water supplemented with chitin, or peptone, or peptone plus yeast extract, or 0.1% (w/v) N-acetylglucosamine showed remarkable changes in their microbial community. Cultures were incubated for 1 week and observed microscopically after staining with DAPI (Fig. 6A-D) and FISH (Fig. 6E and F). Elbe batch cultures fed with raw river water were characterized by a great amount of different green algae and a variety of bacteria of different morphology, as demonstrated in Fig. 6A. Elbe batch cultures supplemented with chitin showed an immense decrease of algae and were dominated by a network of thick filamentous structures (Fig. 6B). Elbe batch cultures supplemented with peptone or peptone plus yeast extract were similar to each other and characterized by a compact structure composed of green algae, diatoms and bacteria of different morphology (Fig. 6C). In N-acetylglucosamine fed batch cultures the occurrence of

algae was rare and the amount of rod shaped bacteria of different sizes increased dramatically after 1 week of incubation (Fig. 6D). Hybridization with specific probes revealed the majority of these rod shaped bacteria as members of the beta-*Proteobacteria.* As demonstrated in Fig. 6E the majority of these cells gave positive hybridization signals with probe beta8b. About 50% within this phylum could be hybridized with the newly developed strain specific probe for beta strain 21. Hybridization visualized unique small rod shaped cells with typical inclusion bodies (Fig 6F). Although all individual samples were hybridized with a variety of domain, class and group specific probes at the beginning of and after one week of incubation no other substrate than N-acetylglucosamine led to a preferred increase of a bacterial phylum as revealed by FISH.

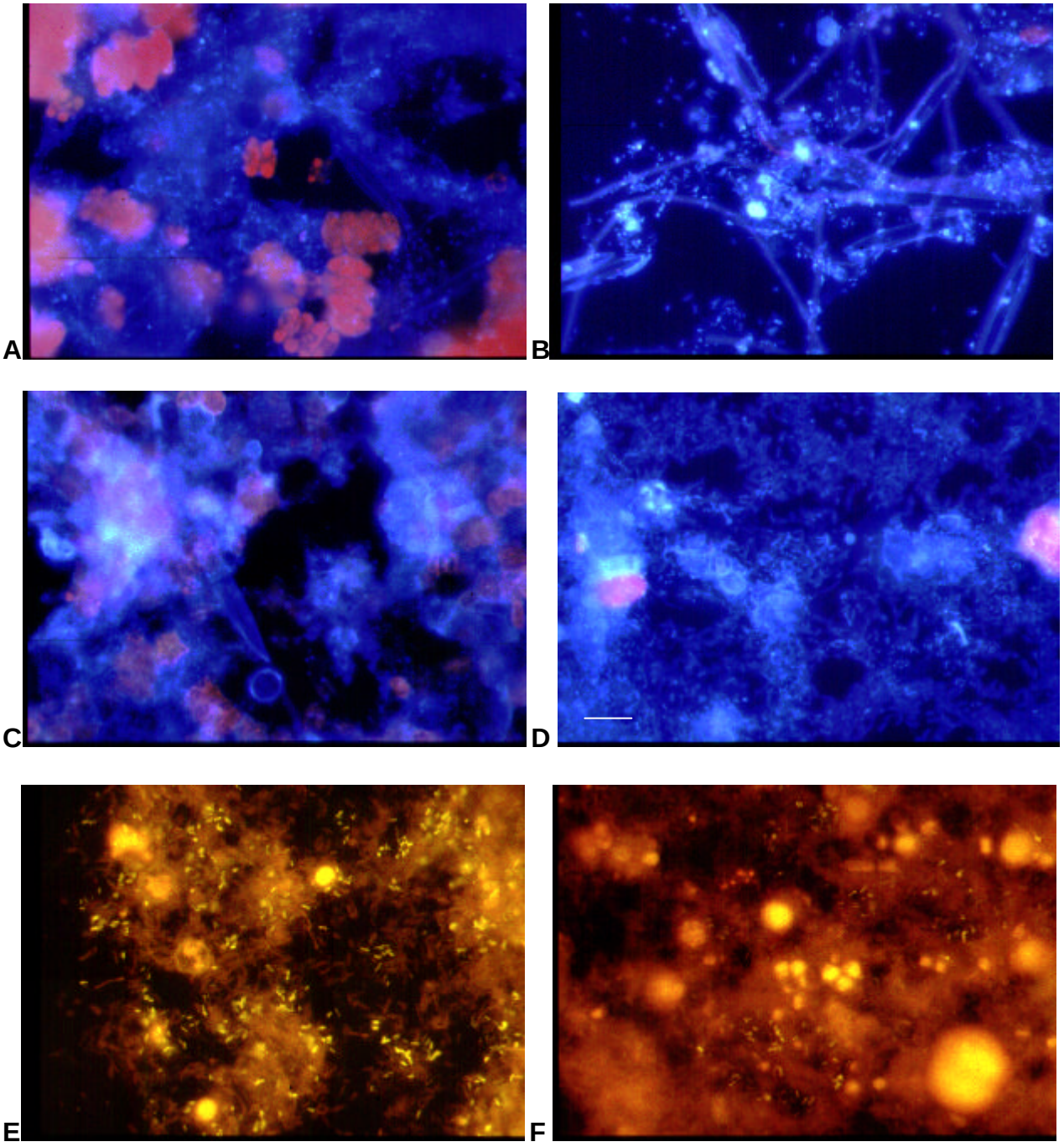


Fig. 6. DAPI staining and FISH photomicrographs of Elbe river snow batch cultures supplemented with various sustrates. **A**: Elbe river snow batch culture fed with raw river water without supplements. **B**: Elbe river snow batch culture supplemented with chitin. **C**: Elbe river snow batch culture supplemented with peptone and yeast extract. **D**: Elbe river snow batch culture supplemented with N-acetylglucosamine. **E**: Elbe river snow batch culture supplemented with N-acetylglucosamine after specific hybridization with probe beta8b. **F**: Elbe river snow batch culture supplemented with N-acetylglucosamine after specific hybridization with probe BETA21. All micrographs were done at 1000 x magnification, scale bar given in D equals 10 µm and is representative for all micrographs.

3.9. *Investigation of extracellular polymeric substances (EPS) as a structural aspect of river snow*

The procaryotic and eucaryotic organisms of the Elbe river snow produced a variety of extracellular polymeric substances (EPS). These formed biofilm matrices and were mainly responsible for aggregate cohesion. Whereas cellular components were detectable with specific oligonucleotide probes (FISH), non cellular components as EPS could be observed with specific fluorescently labelled lectins (LBA: lectin-binding-analysis). In this study FISH and LBA were employed in combination to visualize cellular components and glycoconjugates within natural samples of Elbe river snow.

3.9.1. *Evaluation of the optimal FISH-LBA procedure*

In order to avoid the destruction of the native river snow structure e. g. by centrifugation, in this study river snow aggregates were collected directly from the bulk water phase into coverslip chambers, in which the complete procedure including 1) sample fixation, 2) hybridization, 3) lectin-staining and 4) analysis by confocal laser scanning microscopy was performed. The complete procedure is demonstrated in the flow chart of Fig. 7. Formaldehyd fixation of the aggregates had only a negligible influence on the floc structure, revealed by repeated CLSM analysis of flocs before and after fixation (data not shown). Conventional embedding procedures, e.g. in agarose resulted in weak probe conferred signals after in situ hybridization of the embedded river snow samples (data not shown).

To obtain optimal fluorescent signals by lectin-staining along with strong hybridization signals as demonstrated in Fig. 9A-L, the complete treatment had to be started with the fixation of the sample, followed by FISH and finalized with the

application of fluorescently labelled lectins. Lectin-binding prior to fixation and hybridization, or fixation followed by simultanous hybridization and lectin-staining, resulted in all cases in significantly weaker probe conferred signals and unspecific lectin-binding provoking strong background fluorescence. Post treatment DAPI-staining showed no negative influence neither on probe conferred fluorescence (data not shown) nor lectin signal intensities (Fig. 9E).

The most important precautions of the treatment were (i) the thorough extraction of the liquid component of the sample in order to remove all residual chemicals (e.g. formaldehyde, ethanol), (ii) the evaluation of the optimal working concentration for each lectin, and (iii) the careful removal of unbound lectins. The most effective working concentrations of the fluorescently labelled lectins from *Triticum vulgaris* and *Arachis hypogae* ranged from 50 to 100 ng μl^{-1} protein, whereas lectins from *Limulus polyphemus* and *Phaseolus vulgaris* gave sharper and clearer signals when applied at lower concentrations (10 to 50 ng μl^{-1}). For the *Pseudomonas aeruginosa* lectin, concentrations between 1 and 10 ng μl^{-1} were sufficient.

Step 1: Collect the river snow sample directly into fluid coverslip chambers (100 µl per each chamber), remove the major part of the liquid phase carefully using filtertips leaving a rest of liquid to avoid dehydration of the river snow

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Step 2: Fix the individual river snow sample with 200 µl 3.7% formaldehyde solution for 30 min at 4°C, remove the formaldehyde solution carefully using filtertips

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Step 3: Dehydrate the sample with increasing concentrations of ethanol (50%, 80% and 96% ethanol [v/v]), 100 µl, 3 min each), remove the ethanol solution carefully using filtertips after each ethanol step

–

Step 4: Hybridize the sample for at least 90 min at 46°C in the dark, use 100 µl oligonucleotide probe working solution (probe concentration 5 ng μl^{-1}), remove the liquid phase carefully using filtertips leaving a rest of liquid to avoid dehydration of the river snow

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Step 5: Wash the hybridized sample twice with 500 µl prewarmed washing buffer (46°C) for 20 min and 5 min, respectively, at 46°C in the dark. The volume of the washing buffer used may vary between 500 µl and 1000 µl, remove the liquid phase carefully using filtertips leaving a rest of liquid to avoid dehydration of the river snow

–

Step 6: Stain the sample with 100 µl fluorescently labelled lectin solution for 20 min at room temperature in the dark, the lectin concentration may vary between 1 and 100 ng μl^{1}, remove the liquid phase carefully using filtertips leaving a rest of liquid to avoid dehydration of the river snow

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Step 7: Wash the sample thoroughly at least 5 times with washing buffer, the washing buffer volume may vary between 500 µl and 1000 µl, remove the liquid phase carefully using filtertips leaving a rest of liquid to avoid dehydration of the river snow

–

Step 8: Subject the sample directly to microscopical analysis or store samples frozen at –20°

Fig. 7. Flow chart of the experimental procedure for combined fluorescent in situ hybridization and lectin-binding-analysis (FISH-LBA) showing single steps from sample collection, fixation, in situ hybridization to lectin-binding-analysis.

3.9.2. *Lectin blocking assays*

As shown in Fig. 8 binding ability of lectin from *Limulus polyphemus* was reduced by preincubation of the lectin with both lectin-specific carbohydrates D-glucuronic acid and N-acetylneuraminic acid. For an optimal lectin signal without carbohydrate competition a photomultiplier sensitivity of 630 V was necessary to image the bacterial isolate 18. Preincubation of the lectin with increasing carbohydrate concentrations required increasing voltages/sensitivity of the photomultiplier to obtain adequate lectin signals (up to 703 V for D- glucuronic acid and up to 735 V for N-acetylneuraminic acid).

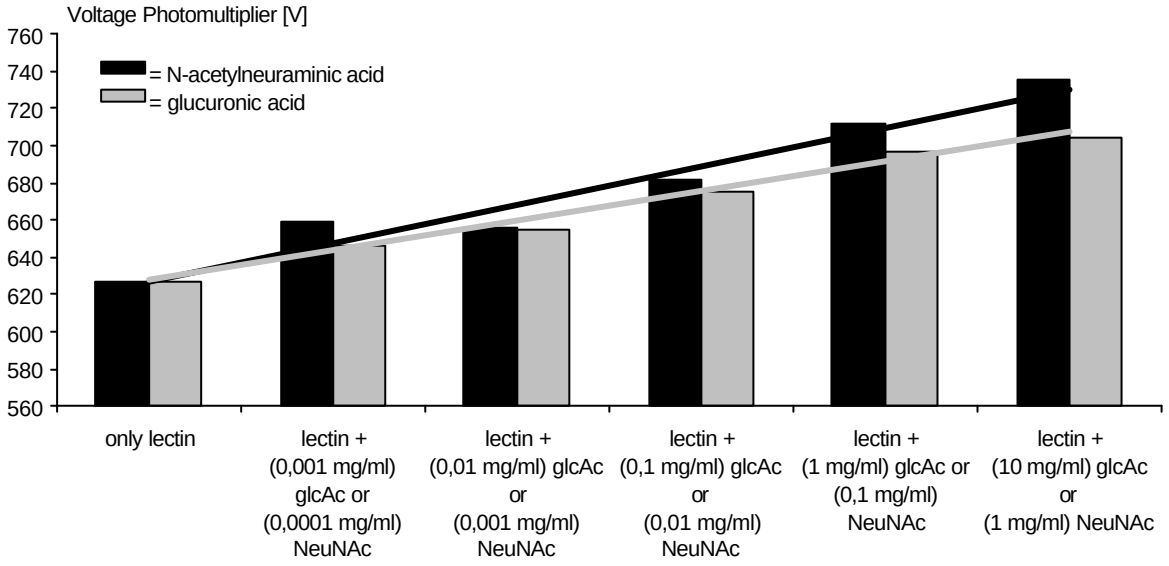

Fig. 8. Lectin blocking assay with lectin from *Limulus polyphemus* and the lectin specific carbohydrates D-glucuronic acid and N-acetylneuraminic acid. The lectin was preincubated for 15 min with increasing carbohydrate concentrations, before staining the bacterial isolate strain 18 (Böckelmann et al. 2000). The figure shows the different voltages of the photomultiplier of the CLSM required for an optimal lectin signal. All values are presented as means of three independant measurements.

3.9.3. *Structural analysis of native Elbe river snow*

The phase contrast micrograph of native Elbe river snow obtained in summer visualized the dominance of a variety of phototrophic organisms (Fig. 9A). Confocal laser scanning microscopy in the reflection mode demonstrated the high mineral content of the lotic aggregates in summer and the skeletons of different diatoms could be observed (Fig. 9B). In Fig. 9C the identical river snow aggregate as given in Fig. 9B is shown after simultaneous staining with the nucleic acid stain SYTO9 and the TRITC-labelled *Triticum vulgaris* lectin binding to NN'-diacetylchitobiose and NN'N''-triacetylchitobiose. The inherent autofluorescence of green algae was simultaneously recorded in the far red channel. Compared with the reflection image (Fig. 9B), the non-homogenous distribution of *Triticum vulgaris* stainable EPS components became visible. It is obvious that the lectin conferred signals showed a more diffuse signal of glycoconjugates, whereas SYTO9 stained specific cellular components. This is in agreement with a previous study on glycoconjugate distribution in fully hydrated lotic aggregates (Neu 2000).

3.9.4. *Overall distribution of glycoconjugates within the river snow EPS*

Using the CLSM in the three channel mode, the simultaneous detection of algae (autofluorescence), bacteria (FISH) and glycoconjugates (LBA) could be performed. As shown in Fig. 9D, large parts of the Elbe river snow could be stained with lectin from *Triticum vulgaris*. Lectin binding visualized both a minor part of distinct filamentous structures and a major part of a complex connected matrix. Bacteria of different cell morphology, ranging from small coccoid, rod shaped, tender filamentous to big spirillum-like shape, hybridized with probe EUB338 specific for the majority of the Bacteria. They were closely attached to the surface of the river snow aggregates.

3.9.5. *Visualization of distinct EPS compartments within the river snow matrix*

Fig. 9E shows an example of simultaneous nucleic acid stained bacteria using DAPI, monitoring of algal autofluorescence and visualization of certain glycoconjugate components of the EPS by staining with lectin from *Pseudomonas aeruginosa*. Although a large number of morphologically uniform filamentous bacteria could be observed, the *Pseudomonas aeruginosa* lectin stained specifically the cell-envelopes along a bundle of certain filamentous bacteria.

Typical representatives of filamentous beta-*Proteobacteria* were detected by in situ hybridization with probe BETA42a in Elbe river snow samples obtained in autumn, as shown in Fig. 9F. Simultaneous staining with fluorescently labelled lectin from *Arachis hypogaea*, binding to D (+) galactose and α-lactose, visualized a slime layer, surrounding a chain of morphologically uniform bacterial cells. Each individual cell gave a probe conferred signal and the whole polysaccharide matrix became visible after binding to the lectin.

3.9.6. *Visualization of eucaryotic and procaryotic cell-envelopes and EPS associated with microcolonies*

The potential of the new method to visualize glycoconjugate components of the EPS on the single cell level could also be demonstrated for algal species. A green algae displaying a distinct morphology in the Elbe summer river snow is shown in Fig. 9G. This algae is typically surrounded by a slime-layer which is extended into radial threads stainable with lectin from *Pseudomonas aeruginosa*. In Fig. 9H, cell clusters of the green algae *Scenedesmus spp*. are shown, that are covered by a thick slime layer, which could be specifically stained with the FITC-labelled lectin from

Pseudomonas aeruginosa. Furthermore, small rod shaped bacteria, hybridized with probe EUB338, were closely attached to and embedded within the lectin-stained EPS.

FISH-LBA analysis was not only successful in the visualization of bacterial EPS components on the level of the whole floc structure and distinct areas within the aggregates, but also showed certain EPS structures surrounding microcolonies in their nearly undisturbed state. Perfoming FISH in coverslip chambers as described here, revealed the frequent occurrence of microcolony forming bacteria which could be observed in spring and summer river snow aggregates (see Fig 9I and J), but not in autumn and winter samples. FISH-LBA with probe EUB338, and FITC-labelled lectin from *Phaseolus vulgaris* which has no specific affinity for any well known sugar, visualized an assemblage of uniform, thick rod shaped bacteria. These bacteria emitted strong probe conferred signals and lectin conferred fluorescent signals located at the cell surfaces (Fig. 9I). All other hybridized cells of different morphology within the microbial aggregates gave no lectin conferred signal. Fig. 9J visualized bacterial microcolonies in the river snow aggregates. Both microcolonies could be successfully hybridized with probe EUB338 and stained with *Phaseolus vulgaris* lectin. However, one microcolony showed a capsule-like structure, whereas in the other microcolony the lectin signal occurred exclusively in the interspace between the individual cells.

In contrast to all other micrographs Fig. 9K and L show a pure culture of the Elbe river snow isolate strain 18. This bacterial isolate is affiliated to the beta-*Proteobacteria,* which were the dominant phylogenetic group in the Elbe samples throughout the whole year. The cell surface of each hybridized cell could be simultaneously stained with the fluorescent *Limulus polyphemus* lectin, binding for example to D-glucuronic acid and/or N-acetylneuraminic acid.

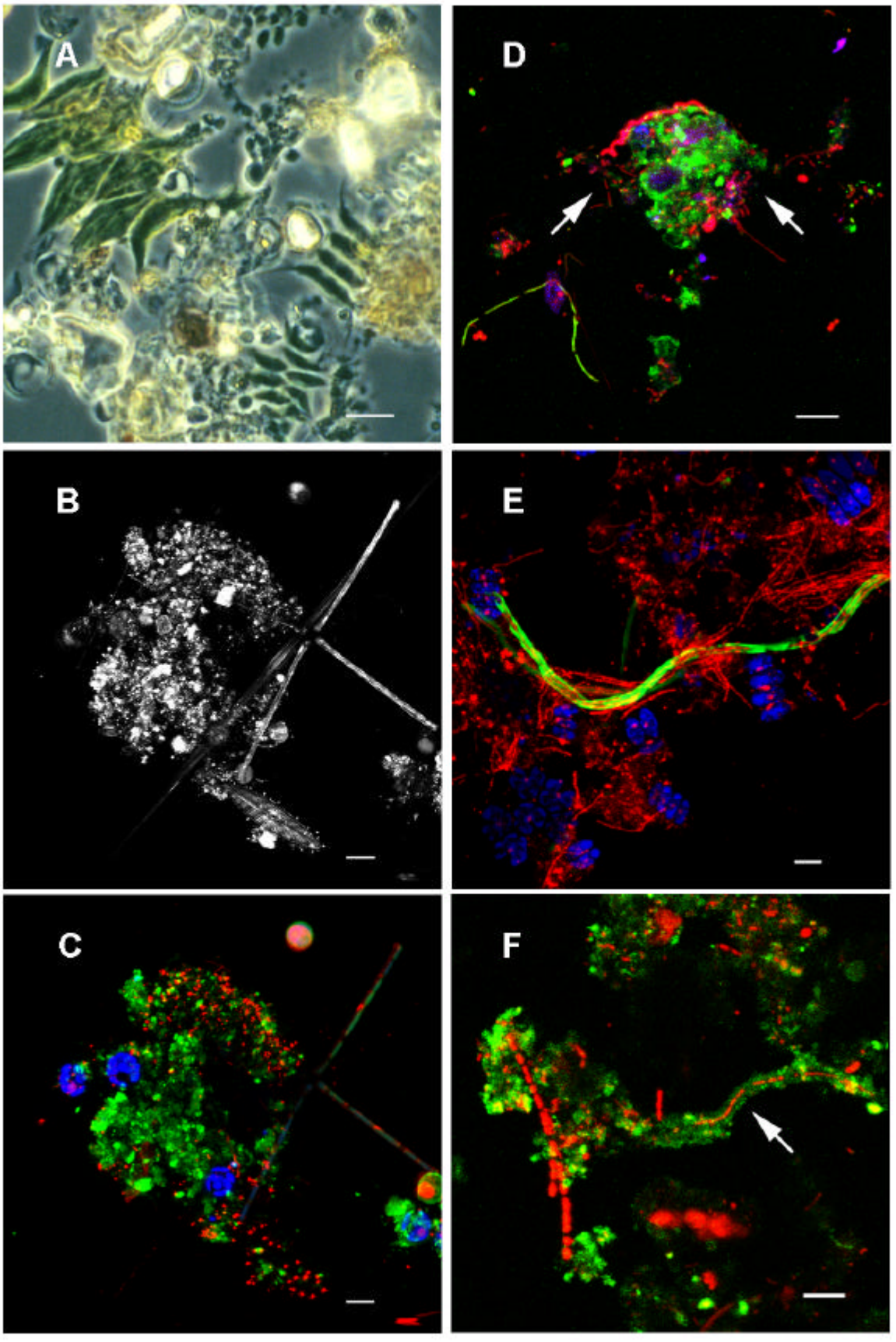

Fig. 9A-F

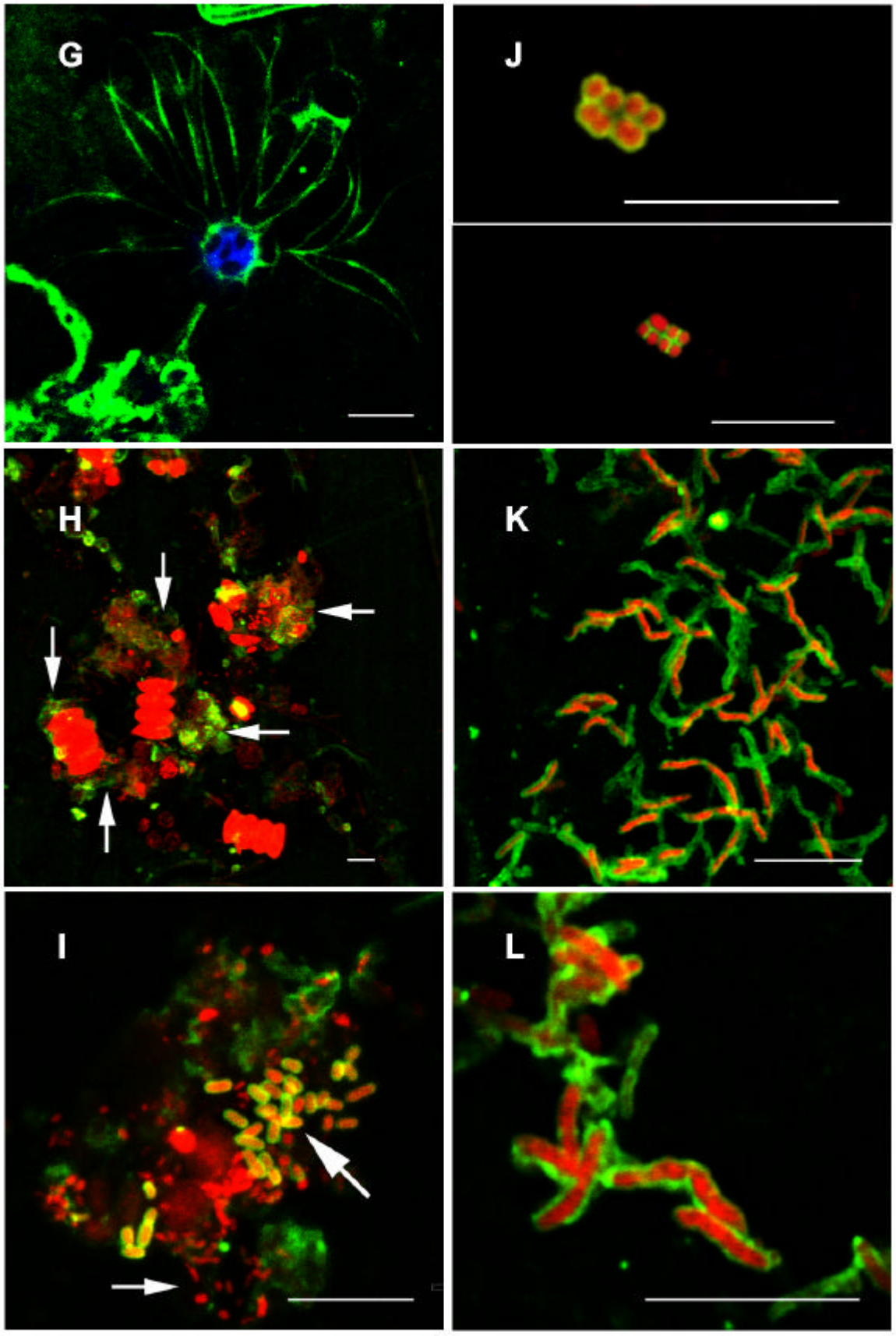

Fig. 9 G-L

Fig. 9.

Micrographs of river snow obtained from the river Elbe at different scales starting from the whole aggregate down to the single cell level. CLSM images are presented as maximum intensity projections. The details of false colour allocation are given with each image. Note the minimal overlap between the different channels and the nearly complete absence of non-specific staining. Each scale bar indicates 10 µm.

(A) Phase contrast micrograph of Elbe river snow collected in summer showing the dominance of various algae.

(B) CLSM micrograph of the reflection signal from an unfixed, fully hydrated Elbe river snow aggregate obtained in summer, demonstrating mineral content of geogenic and biogenic origin.

(C) The same native lotic aggregate as in (B) collected in the CLSM fluorescence mode. False colour allocation in this image is staining with SYTO9 (red) and the *Triticum vulgaris* TRITC-lectin (green). Far red autofluorescence of green algae was also recorded (blue).

(D) CLSM micrograph of a fixed lotic aggregate of the Elbe river collected in summer. The sample was hybridized with probe EUB338 (red) specifically binding to the majority of the domain *Bacteria*, and stained with *Triticum vulgaris* FITC-lectin (green). Note the different morphologies of bacteria attached to the floc surface (indicated by arrows).

(E) CLSM micrograph of an enlarged area within a fixed Elbe river snow collected in summer. The sample was stained with DAPI (red) and *Pseudomonas aeruginosa* FITC-lectin (green). Autofluorescence of various green algae is also shown (blue). Note the very specific glycoconjugate labeling along a bundle of filamentous bacteria.

(F) CLSM micrograph of an enlarged area within a fixed Elbe river snow collected in autumn. The sample was hybridized with probe BETA42a specific for the beta-subclass of *Proteobacteria* (red) and stained with *Arachis hypogaea* FITC-lectin (green). Note the chains of morphologically uniform hybridized bacterial cells characteristic for the sheathed bacteria and the thick lectin-stained slime-layer around them (indicated by arrow).

(G) CLSM micrograph of a representative green algae from a fixed Elbe river snow collected in summer. The chlorophyll autofluorescence (blue) is surrounded by radial threads of glycoconjugates stained with *Pseudomonas aeruginosa* lectin.

(H) CLSM micrograph of a fixed Elbe river snow obtained in summer with green algae of the species *Scenedesmus* associated with stained polymers. False colour

allocation in this image is red for algal autofluorescence (large cells/area) as well as for hybridization signal (small cells) and green for glycoconjugate. Note the bacteria (hybridized with probe EUB338) embedded in the *Pseudomonas aeruginosa* FITC-lectin stained EPS material (indicated by arrows).

(I) CLSM micrograph of an area within a fixed Elbe river snow obtained in summer. The sample was hybridized with probe EUB338 (red) and stained with *Phaseolus vulgaris* FITC-lectin (green). Note that only the cell-envelopes of the uniform thick bacterial cells could be stained with the lectin (large arrow). Other hybridized cells of different morphology gave no lectin signals (small arrow).

(J) CLSM close-up of two bacterial microcolonies from a fixed Elbe river snow collected in summer after hybridization with probe EUB338 (red) and staining with Phase*olus vulgaris* FITC-lectin (green). Note that the entire cell surface of the upper microcolony is covered by a lectin-stained capsule, whereas the lower microcolony showed the lectin signal only in between the cells.

(K) CLSM micrograph of an overview of a pure fixed culture isolated from Elbe river snow affiliated to the beta-subclass of *Proteobacteria* and hybridized with probe EUB338 (red). Glycoconjugates were stained with *Limulus polyphemus* FITC-lectin (green).

(L) CLSM micrograph of an enlarged area of the same sample as in (K). Note the clear hybridization signal within and the glycoconjugate labeling of cell surface polymers obtained by the combined technique of FISH and lectin-binding-analysis.

3.10. *Description of the unusual cell organization behaviour of river snow isolate F8*

Following the cultivation strategy of oligotrophic low nutrient media for the isolation of in situ relevant Elbe river snow bacteria, in summer 1999 river snow samples of the Canadian South Saskatchewan River were investigated. Cultivation led to the discovery of the bacterial strain F8. Strain F8 was generally characterized by a remarkable kind of cell-cell interaction along a self-produced filamentous network.

3.10.1. *Isolation and phylogenetic affiliation of strain F8*

In total 36 different bacterial strains were isolated from the South Saskatchewan river snow samples and further investigated at the National Water Research Institute in Saskatoon, Canada. One of them, strain F8 was isolated on FBM medium on which it formed light yellow colonies of 1-2 mm in diameter. The colonies were adherent to the agar surface and were difficult to transfer. As shown in Fig. 10 phylogenetic analysis revealed strain F8 as a deep branching gamma-*Proteobacterium* with just a small number of known relatives. The closest relative to strain F8 was *Curacaobacter baltica*, (96.1% sequence identity), a bacterium isolated in the central Baltic and able to produce a striking Curacao-blue unpolar pigment (Strobowski et al. unpublished data). Strain F8 was further closely related to the unidentified bacterium 38 with a sequence identity of 95.8% and the unidentified bacterium 30 (sequence identity of 92.1%). These bacteria were isolated from aquatic sources of drillwater and a soda lake in Sweden (Duckworth et al. unpublished data). No similar behaviour was reported for any of these relatives of F8.

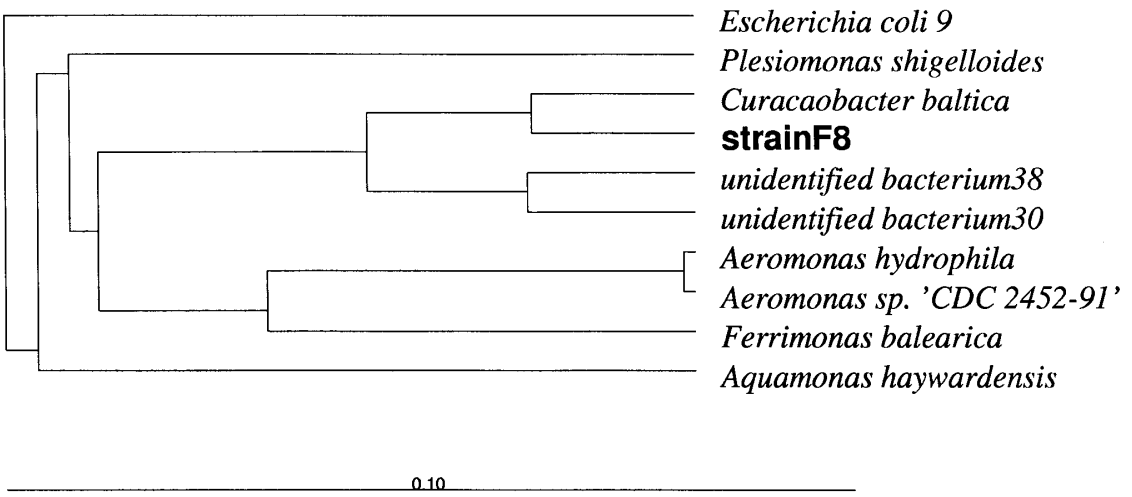

FIG. 10. Phylogenetic tree inferred from 16S rRNA sequence data, showing the relationships of strain F8 affiliated to the gamma-*Proteobacteria* to its closest known relatives: The tree was reconstructed by the neighbor-joining method, and the scale bar represents 10 nucleotide substitutions per 100 nucleotides.

3.10.2. *Physiological potential and the ability of filament formation of isolate F8 on different media*

The bacterium F8 was initially isolated from the oligotrophic freshwater basal medium. Carbon utilization analysis (see Table 6) indicated that the organism was not fastidious. Strain F8 grew on a variety of substrates, and although affiliated to the gamma-*Proteobacteria,* it showed a quite different BIOLOG pattern compared with the Elbe gamma-*Proteobacteria* isolates. In contrast to the Elbe isolates strain F8 was able to utilize the majority of the carbohydrates including D-psicose. Utilization of carboxicacids was as good as carbohydrates including alpha-ketobutyric acid and alpha-ketovaleric acid, which both could not be utilized by any of the Elbe gamma-*Proteobacteria* isolates. Obviously, aminoacids were not the preferred substrate for strain F8, whereas most of the Elbe gamma-*Proteobacteria* isolates were able to degrade the majority of the aminoacids. In agreement with all Elbe river snow isolates strain F8 was able to use polymers and polyethylene-sorbitane as substrate. Strain F8 utilization of ester, alcohols, amids, amins, phosphorylated and aromatic compounds was diverse in contrast to the Elbe gamma-*Proteobacteria* isolates. Bromosuccinic acid, which could be degraded by all Elbe gamma-*Proteobacteria* isolates and the majority of all other Elbe river snow isolates did not serve as substrate for strain F8. Although the isolate was able to grow on/in a variety of different media, the entire process of filament formation was not observed on/in all media with equal intensity. Results are summarized in Table 7.

TABLE 7. Growth and the ability of filament formation of isolate F8 on different solid media

Medium	Growth	Cell morphology	Filament formation
TSA	weak, even after 1 week	large rod shaped cells (2-3 µm)	no
TSA 10%	good, overnight	rod shaped cells (~1 µm)	yes
R2A	good, overnight	rod shaped cells (1-2 µm) often in small or long chains	no
CD	weak, after 2-3 days	very large cells (up to 5 µm) of unregular morphology	no
CF	good, overnight	rod shaped cells (~1 µm)	yes
FBM	good, after 1-2 days	rod shaped cells (~1 µm)	yes
RRW a	weak, after 2-3 days	rod shaped cells (~1 µm)	yes
RRW b	weak, after 2-3 days	rod shaped cells (~1 µm)	yes

TSA: Tryptic soy agar, soybean-casein digest medium
R2A: medium for heterotrophic organisms in treated potable water
CD: Czapek-Dox medium
CF: medium for Cytophaga and Flavobacteria
FBM: freshwater basal medium
RRW a: raw river water of the South Saskatchewan River supplemented with 100 µl l^{-1}glucose stock solution (20 g l^{-1});
RRW b: raw river water of the South Saskatchewan River supplemented with 100 µl $l^{-1;}$ each glucose-, ammonium chloride (45 g l^{-1})- and phosphate (11 g l^{-1})-stock solutions.

In terms of growth strain F8 preferred low nutrient media as indicated by no growth on NB medium, and only weak growth on full TSA medium. In terms of behaviour no filament formation was observed on full strength media. In contrast cultivation on 10% TSA led to fast growth combined with the formation of filaments. The fastest growth of isolate F8 was observed on R2A and CF medium. But, filament formation occurred only on CF medium. The amount of filaments on CF was high, compared with that observed on RRW media. However, the most extensive filament production was observed when F8 was cultured on/in FBM medium. A filament free F8 culture grown on R2A medium was still able to produce filaments, after transferring it to FBM medium. Filaments could be observed after 2 - 4 days of incubation in this medium indicating that nutrition played a key role in expression of this behavioural trait in F8.

3.10.3. *Histochemical characterization of the filaments*

In order to get additional informations regarding the nature of the filaments a great variety of different dyes was used to stain the bacterial cells and filaments. The results are summarized in Table 8. Filaments could be visualized with Methylene blue and more weakly with tannic acid, a flagella protein stain. In contrast to this, the protein stains SYPRORed and SYPROOrange gave no signals with the filaments. Although the lipid stain NileRed successfully stained the bacterial cell surfaces, it did not stain filaments. All of the 17 lectins used were unable to bind to the filaments implying that they possibly do not consist of carbohydrates or polysaccharides. Five of the lectins (*Arachis hypogaea, Conavalin A, Tetragonolobus purpurea, Triticum vulgaris* and *Wisteria floribunda*) stained the bacterial cell surfaces, of which *Concanavalin A* worked best. Interestingly, optimal filament detection could be observed after staining with the DNA specific stains SYTOXGreen, SYTO9, SYTO62 and SYTO63.

TABLE 8. Characterization of isolate F8 by different staining methods

General dye	Specificity	stains cells	stains filaments
Ink	whole cell	no	no
Crystal violet	cell wall	no	no
Methylene blue	whole cell	yes	**yes**
Carbolfuchsin	acid resistant bacteria	weakly	no
Methyl Red	whole cell	no	no
Ruthenium Red	whole cell	yes	no
Congo Red	whole cell	yes	no
Malachite Green	endospores	no	no
tannic acid	flagella (protein)	yes	**yes**
Fluorescent dyes			
NileRed	lipids	yes	no
SYPROOrange	proteins	yes	no
SYPRORed	proteins	yes	no
Dextran 10000 MW	whole cell	no	no
Dextran 70000 MW	whole cell	no	no
BODIPY	cell membranes	no	no
DAPI	DNA	weakly	no
SYTOXGreen	DNA	yes	**yes**
SYTO9	DNA	yes	**yes**
SYTO15	DNA	yes	no
SYTO62	DNA	yes	**yes**
SYTO63	DNA	yes	**yes**
Fluorescently labelled lectins	extracellular polymeric substances		
Arachis hypogaea	β-gal(1-3) galNAc	yes	no
Bandeiraea simplicifolia (BS-I)	α-gal, α-galNAc	no	no
Caragana arborescens	galNAc	no	no
Concanavalin A (ConA)	α-man, α-glc	yes	no
Erythrina cristagalli	β-gal(1-4) glcNAc	no	no
Glycine max	galNAc	no	no
Lens culinaris	α-man	no	no
Limulus poyphemus	NeuNAc, galNAc, glcNAc	no	no
Lycopersicon esculentum	$(glcNAc)_3$	no	no
Pseudomonas aeruginosa (PA-I)	gal	no	no
Tetragonolobus purpureas	α-L-fuc	yes	no
Triticum vulgaris (WGA)	$(glcNAc)_2$, NeuNAc	yes	no
Ulex europaeus (UEA-I)	α-L-fuc	no	no
Ulex europaeus (UEA-II)	$(glcNAc)_2$	no	no
Vicia faba	man, glc	no	no
Vicia villosa	galNAc	no	no
Wisteria floribunda	galNAc	yes	no

The general dyes were examined with bright field microscopy.
The fluorescent stains were examined by epifluorescence or confocal laser scanning microscopy. The lectins were used FITC or TRITC labelled and examined by confocal laser scanning microscopy.

Staining with SYTOXGreen resulted in best filament visualization compared with all other stains applied. Fig. 11A and 11B show the sponge like strucure of the filamentous network formed by strain F8 stained with SYTOXGreen. Fig. 11C shows cells and filaments of isolate F8 after double staining with SYTOXGreen and NileRed. Filament detection with the nucleic acid stains SYTO9, SYTO62 and SYTO63 was equally. In contrast to these stains SYTO15 and DAPI were not able to stain the abundant filaments.

3.10.4. *Proof of filaments abundance by different microscopical analysis*

Phase contrast microscopy proved to be an unsuitable method to visualize the translucent cells of strain F8 and its filigree filaments. Although cells were visible, filaments could never be observed. In contrast, epifluorescence- and confocal laser scanning-microscopy were both suitable tools to visualize bacterial cells and filaments (see Fig. 11A-C) if effective fluorescent stains were employed. In order to avoid pressure on the filament material (using a cover slide for microscopy), which might cause disruption of the network and other artefacts, a complete bacterial colony of strain F8 was transferred from solid medium into a special coverslip chamber. After staining with SYTO9 and inverted microscopical analysis, the sponge like structure could be observed (data not shown). In agreement with the CLSM images, transmission electron microscopy (TEM) of F8 cells grown in liquid or solid culture, revealed the abundance of an unusual fuzzy filamentous material, which surrounded the cell surfaces of the Gram-negative strain F8 (Fig. 11D). TEM images showed small round structures of different sizes budding from the cell surfaces and surrounded by a membrane. In agreement with the CLSM observations these structures connect with each other to elongate and to get a filamentous shape (Fig. 11E).

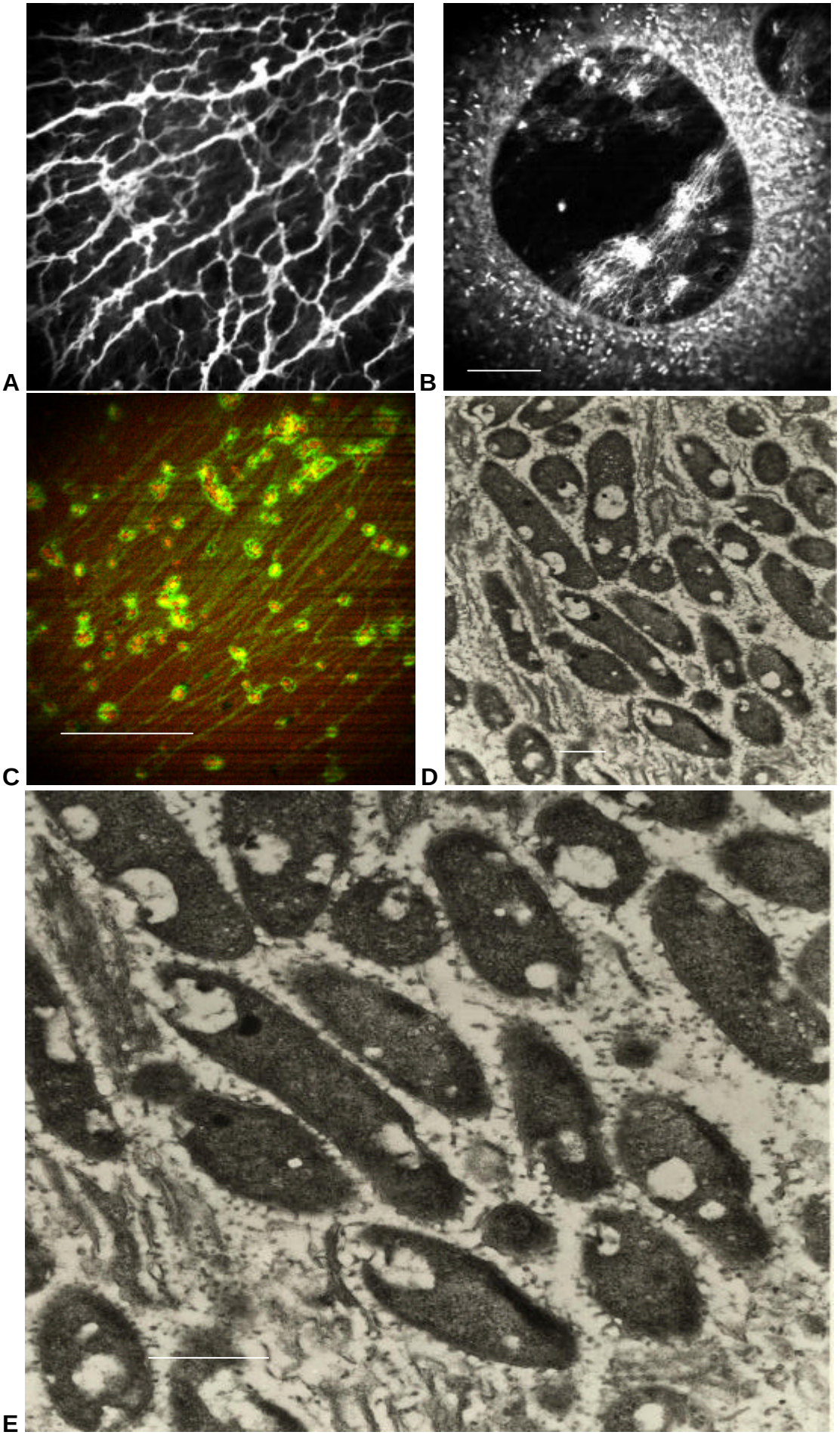

Fig. 11A-E.

Fig. 11A-E. CLSM and TEM micrographs of strain F8 growing in liquid culture in FBM medium. **A:** CLSM micrograph of the filamentous network of an unfixed native F8 liquid culture after staining with SYTOXGreen. **B:** CLSM micrograph of the sponge like structure of an unfixed native F8 liquid culture after staining with SYTOXGreen. Scale bar equals 10 µm and corresponds to micrographs A and B. C: CLSM micrograph of an unfixed native F8 liquid culture after double staining with SYTOXGreen and NileRed. Scale bar equals 10 µm. **D:** TEM micrograph of a fixed F8 liquid culture showing single cells with filamentous material around them. Scale bar equals 1 µm. **E:** TEM micrograph of a fixed F8 liquid culture showing single cells and small structures in the space in between varying from small round to filamentous shaped structures. Scale bar equals 1 µm.

3.10.5. *The 4 step process of filament formation of strain F8*

The complete process of filament formation involved several steps which are shown in Fig. 12A-H. In the first step the rod shaped bacterial cells accumulated a self-produced material around them. This material appeared amorphous and particulate in nature and accumulated around the cells increasing apparently the diameter several fold (Fig.12A and B). In the second step the material changed into thin filigree filaments of initially short and subsequently longer size. The cells remained associated with the filaments, while the filaments became interconnected and the cells subsequently connected with other cells by moving along the interconnecting filaments (Fig. 12C and D). In the third step single cells joined together with other single cells assembling small and subsequently larger cell-aggregates. Filament formation and cell aggregation developed into a complexe structure with a sponge-like apparence, where areas of cell clusters alternated with cell free spaces (Fig. 12E and F). At the end of the process a combination of large cell clusters without filaments and remaining empty filaments without cells could be observed (Fig. 12G and H).

Fig. 12. Epifluorescence micrographs of native bacterial cells of strain F8 grown in FBM liquid medium after staining with SYTO9. The figure shows the complete process of cell organization. **A** and **B**: rod shaped bacterial cells accumulating a self-produced material around them. **C** and **D**: material elongation, formation of fibres, cells start moving along the fibres**. E** and **F**: formation of a filamentous network, cells keep on moving. **G** and **H**: formation of large cell aggregates and remaining empty filaments without cells. All micrographs were done at 1000 x magnification and the scale bar in H equals 10 µm and is representative for all other micrographs.

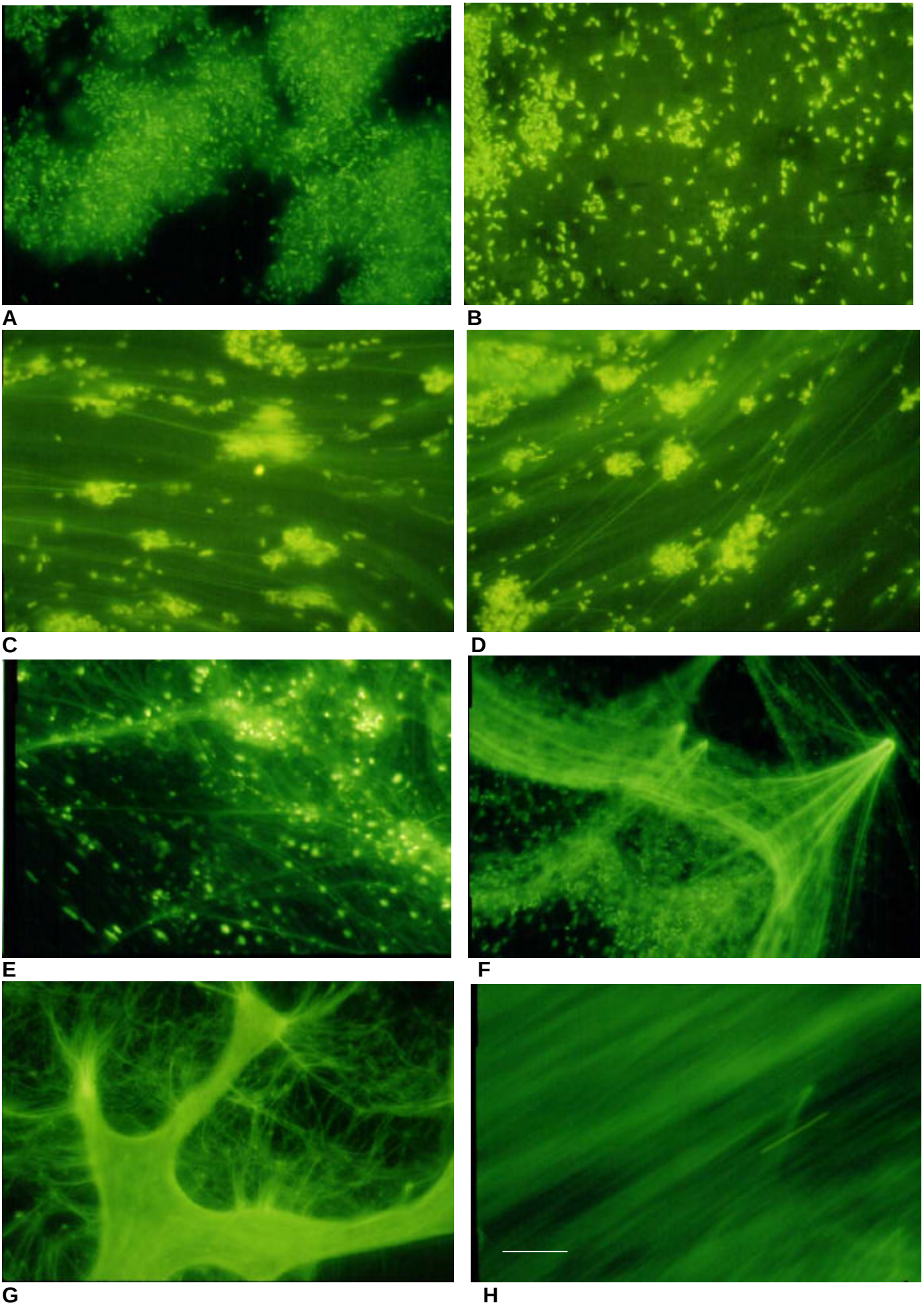

Fig. 12A-H

4.Discussion

4.1. Lotic microbial aggregates

Much of the suspended organic and inorganic matter in aquatic systems exists as aggregates, which range from a few microns to many centimeters in size. Aggregates are ubiquitous in oceans (Azam et al. 1993, Delong et al. 1993, Herndl 1988, Simon et al. 1990, Smith et al. 1992), lakes (Grossart 1995, Grossart & Simon 1993, Weiss et al. 1996), estuaries (Eisma 1992, Zimmermann 1997, Zimmermann & Kausch 1996), and rivers (Böckelmann et al. 2000, Neu 2000). Aggregates are rich in nutrients, particularly those containing carbon, which enhances their colonization and the subsequent growth of the organisms. They are characterized by their bacterial content, which is two or three orders of magnitude greater than that in surrounding water (Caron et al. 1982, Müller-Niklas et al. 1994). For that reason the biological study presented in this work has focused mainly on the bacterial community. Studies in marine and freshwater habitats have shown that aggregates play an important role in the flow of energy through microbial communities as well as in horizontal (Berger et al. 1996) and vertical transport (Turley & Carstens 1991) of nutrients in riverine systems. Aggregates of marine and lake snow flocs are fragile and amorphous in microstructure. In contrast to this, lotic aggregates are exposed to permanent shear forces created by turbulent flow and thereby may have a more stabile structure. Rivers are influenced by a variety of ecological, hydrological and geomorphological processes (Allan 1995). Ecologists, who study streams should consider anthropogenic modifications of the stream and its valley as well as the heterogenity of a stream in general. If they do not contribute this to their observations, they do so at the risk of incomplete understanding. Furthermore scientists working on lotic microbial aggregates should realize (i) that sampling and any further treatment might

destroy their native state and (ii) that each sample is different to all the others. Nevertheless the results of the present study show that river snow samples collected in one season are similar to each other but they differ from season to season. The seasonal variations of the river snow aggregates in terms of the structure and composition of the microbial community shown in this study are in good agreement with the results of Zimmermann (1997), who investigated the microbial community of aggregates in the Elbe estuary. Zimmermann examined the aggregate composition, the abundance of protozoa, metazoa and the total cell counts of attached and free bacteria. Aggregates of both sampling sites showed seasonal differences in size, morphology and content. A significant correlation between the abundance of attached bacteria and the size of the aggregates could be demonstrated for both. In the Elbe estuary large particles typically occurred when the organic content was high. During spring time, aggregates of both sampling sides were large and fairly fragile. Elbe estuary aggregates harboured intact diatoms, loricas of tintinnids and faecal pellets of copepods (Zimmermann & Kausch 1996). In this study microscopical analysis of Elbe river snow samples also revealed a high amount of diatoms in spring time. Aggregate fragility was obviously caused by a filamentous network of Cytophaga as shown in Fig. 3C. In agreement with the Elbe estuary during summer, large amounts of detritus, cyanobacteria, and green algae could be observed in the aggregates. The high abundance of algae in the summer river snow community was not correlated with a significant change of the total bacterial cell counts. Total bacterial concentration of both sampling sites was comparatively high in spring and summer and decreased in autumn and winter. The microbial populations of the river snow community in autumn and winter were quite similar to each other. In these cases the decrease in bacterial cell counts might be caused by the lack of nutrients and the low temperatures. Zimmermann showed that the total organic content of particles varied considerably. During the winter, when the water discharge rate was

high, mineral particles were very abundant. Zimmermann (1997) and Wörner et al. (2000) supposed that probably due to the greater resuspension of sand or grains, the number of attached bacteria on those aggregates remained low. Confirming these results the characteristic features of the autumn and winter aggregates at km 322 of the Elbe River were the absence of algae and the more compact structure of the river snow aggregates.

4.2. *Seasonal dynamics of the bacterial Elbe river snow community*

The present study focused on the seasonal distribution of the main phylogenetic bacterial groups within the microbial river snow community. These results are in part contradictory to the results of Brümmer et al. (2000). They investigated the abundance of dominant phylogenetic bacterial groups in biofilms growing on glass plates exposed below the river surface of the Elbe and one of its tributaries, the Spittelwasser. A variety of physiological and biological forces may gover the species composition of biofilms grown on glass plates and river snow with respect to structure and composition. Therefore one should not expect a 100% similarity of the microbial communities.

In spring, the Elbe river snow community at km 322 was characterized by the high relative abundance of cytophaga, which were repeatedly shown to be important members of bacterial river communities (Kenzaka et al. 1998, Leff 1994, Manz et al. 1999). Spring bloom in aquatic environments is often characterized by a strong increase of the population of phyllopods and ciliates, followed by a seasonal maximum of total bacterial cell counts and bacterial production (Schwoerbel 1999). Since cytophaga are able to degrade macromolecules such as chitin, which mostly originates from the skeletons of crustaceans, they may act as important destruents in this scenario. This is corraborated by observations in marine environments, where

cytophaga are abundant on decaying sea animals, such as crustaceans (Reichenbach 1992). Brümmer et al. (2000) observed a bimodal distribution of the abundance of members of the CF cluster in Elbe biofilms with maxima in February and July. Probe CF319a is specific for both the filamentous Cytophaga and the rod shaped Flavobacteria. Unfortunately, Brümmer gave no information about the morphology of hybridized bacteria. If for example filamentous Cytophaga caused the maximum in February whereas rodshaped Flavobacteria produced the maximum in July, this would be more consistent with our results.

In this study, alpha-*Proteobacteria* constituted a minor part of the bacterial river snow population, which is in agreement with their distribution in the Elbe and Spittelwasser biofilms (Brümmer et al. 2000). Similar results were reported from lake snow particles in Lake Constanze, where alpha-*Proteobacteria* composed up to 20% of the aggregate-associated microbial community (Weiss et al. 1996). They are also ubiquitous members of free-living marine bacterioplankton communities (DeLong et al. 1993), microbial communities in marine (Rath 1998) and laboratory made riverine aggregates (formed after incubation of water samples in roller tanks) (Grossart & Ploug 2000). Their highest relative abundance in the Elbe river snow occurred on large loosely arranged aggregates in spring. In situ hybridization of laboratory made riverine aggregates (Grossart & Ploug 2000) revealed a significant decrease of alpha-*Proteobacteria* correlated with aggregate formation and composition. This might indicate both: (i) various aggregates support colonization by different phylogenetic bacteria and (ii) bacterial populations are involved in aggregate development.

In this study and in the study of Brümmer et al. (2000) gamma-*Proteobacteria* did not constitute a major component of the community, based on numerical abundance, neither in the Elbe river snow nor in the Elbe or Spittelwasser biofilms, whereas laboratory made riverine aggregates showed an increasing amount of gamma-

Proteobacteria during incubation time (Grossart & Ploug 2000). Gamma-*Proteobacteria* are distributed throughout a wide range of aquatic habitats, but appear to be more dominant in marine environments (Giovannoni et al. 1995, Hiorns et al. 1997, Nold & Zwart 1998).

In this study the beta-*Proteobacteria* constituted the numerically most abundant bacterial group in the river snow community during all seasons. The relative abundance of beta-*Proteobacteria* ranged from 50% to 54% throughout the year. Although Brümmer et al. (2000) also detected beta-*Proteobacteria* in biofilms of the Elbe and the Spittelwasser throughout the year, there was a significant difference in the average amount of this phylum, only 18% in the Elbe, compared with 31% in the Spittelwasser. The significantly higher ammonia concentrations in the Spittelwasser than in the Elbe might have supported a large population of ammonia-oxidizing bacteria, a monophyletic assemblage in the *beta-Proteobacteria*.

The observed predominance of beta-*Proteobacteria* in our study is in agreement with previous reports on phylogenetic diversity studies in a variety of other freshwater systems, including drinking water biofilms (Kalmbach et al. 1997b, Manz et al. 1993), an oligotrophic high mountain lake (Alfreider et al. 1996), activated sludge (Wagner et al. 1993) and lake snow (Grossart & Simon 1993). In contrast to this, there appears to be a striking absence of these organisms in marine environment (Nold & Zwart 1998) due to their sensitivity towards increasing salinity. As reported by Weiss and colleagues (1996), the microbial lake snow community in the Lake Constance was dominated by beta-*Proteobacteria* during the whole year. In the river snow aggregates of the Elbe, beta-*Proteobacteria* were mostly represented by small, rod shaped bacteria with characteristic inclusion bodies, hybridizing with probe beta8b as shown in Fig 3B. Bacteria of the same typical cell shape could also be hybridized in a study investigating the population structure of lotic bacterial biofilms (Manz et al. 1999). From a morphological and physiological view, the beta-subclass of

Proteobacteria is an extremely heterogenous family with few common features (De Ley 1992). Thus one should not expect a single reason for their dominance in freshwater microbial communities. A variety of physical forces as salinity, depth, resource availability and biological forces as competition, interactions, predation and parasitism are able to govern microbial species distribution. Detailed studies of these forces with respect to beta-*Proteobacteria* are necessary to explain their fitness advantage in comparison with other bacterial species.

The detection of Planctomycetes in this study as members of the river snow community (reaching 2 - 11% of total bacterial cell counts in the course the year) agrees with their widepread occurrence in aquatic habitats (Neef et al. 1998). They have been shown to follow or accompany algal or cyanobacterial blooms (Fuerst 1995). Some Planctomycetes exhibit specialised appandages or stalks used for particle attachment, and all characterized species are capable of aerobic decomposition of polymeric substrates. These results indicate, that specific microbial populations are adapted to the unique niche provided by suspended particles. Fuerst (1995) assumed that these populations might be responsible for particle decomposition in aquatic habitats.

The more compact aggregate structure in autumn and winter might be the reason for the remarkable increase of sulfate-reducing-bacteria, which constituted 17% of all bacteria in autumn and 9% in winter, in contrast to their low relative abundances in spring and summer (2% and 4%, respectively). Strong fluorescent signals after hybridization of the SRB indicated their high metabolic potential in this season. Unexpectedly, the SRB were not restricted to distinct locations within the aggregates. They were equally dispersed throughout the whole river snow structure. These results are in good agreement with Manz et al. (1999), who investigated the phylogenetic composition of lotic bacterial biofilms. They showed, that FISH with a variety of SRB specific probes visualized single SRB equally dispersed throughout

the whole biofilm. SRB are obviously present and metabolic active in aerobic and periodically oxic habitats including drinking water biofilms (Bade et al. 2000), activated sludge (Manz et al. 1998), oxic surface sediment of a seawater lake (Fukui & Takii 1990) or the oligotrophic lake Stechlin (Sass et al. 1997).

4.3. *Cultivation efficiency and in situ relevance of isolated river snow bacteria*

The results of the isolation strategy used in this study emphasize the importance of optimized media to gain maximum cultivation efficiencies for specific bacterial groups. Keeping in mind that the majority of bacteria from aquatic environmental samples is adapted to low nutrient conditions in their natural habitat, oligotrophic media should be preferred. The Elbe river snow isolates described in Table 5 showed a broad range of phylogenetical affiliation. They belonged to the alpha-, beta-, gamma-, and delta-*Proteobacteria*, the *Cytophaga-Flavobacteria Bacteroides* group, firmicutes with low and high GC content of DNA, and firmicutes with atypical cell wall. The sequence similarity to their next known relatives (Fig. 4A-I) also showed a broad range from 99.8% for some Clostridia and gamma-*Proteobacteria* to 89.8% for strain 18, a beta-*Proteobacterium.*

With the exception of strain 26 all of the newly isolated alpha-*Proteobacteria* were cultivated on low nutrient media. These results agreed with their physiological profiles, which were characterized by a minimal utilization of the offered carbon sources. Confirming these findings, Morgan & Dow (1985) regarded most of the genera of the alpha-*Proteobacteria* as oligotrophic, a useful term, when it is not defined too strictly to characterize organisms adapted to life in low nutrient ecosystems. Members of the alpha-*Proteobacteria* show modest growth at very low carbon-source concentrations, a variety of them cannot tolerate nutritional excess. Phylogenetic analysis of the five bacterial river snow isolates affiliated to the alpha-

Proteobacteria revealed the majority of them as closely related to the *Rhizobiaceae* and *Sinorhizobiaceae,* respectively. These are typical soil inhabitants, capable of living in symbiosis with different plants. This might indicate that some of the river snow isolates affiliated to the alpha-*Proteobacteria* are not aquatic bacteria “senso stricto”. Possibly they were washed out from soil, either via surface water or ground water or carried into the water with plant roots. Nevertheless, a striking example for the importance of the isolation of an environmental alpha-*Proteobacterium*, is given by Gonzales & Moran (1997). They investigated the distribution and abundance of alpha-*Proteobacteria* along salinity gradients in coastal rivers using a combination of oligonucleotide probing, SSU rRNA sequence retrieval and cultivation techniques. The abundance of the alpha phylum declined with decreasing salinity until none were detected in freshwater samples. Only the successful cultivation of a marine alpha-*Proteobacterium*, whose SSU rRNA sequence was recovered in a clone libary allowed its physiological characterization and the determination of its tolerance of low ion concentrations.

All of the newly isolated beta-*Proteobacteria* were exclusively cultivated on low nutrient media, disclosing them as oligothrophic bacteria. The in situ relevance of all six isolates could be demonstrated with the newly developed cluster and strain specific probes. Hybridization with probe BETA21 specific for strain 21 revealed this isolate as a dominant member of the bacterial Elbe river snow community throughout the year. Hybridization visualized unique small rod shaped cells with typical inclusion bodies reaching respectively 1% of the total river snow cell counts. This is a remarkable result, keeping in mind, that using conventional cultivation techniques, less than 1% of the bacterial population from oligotrophic systems per se can be cultivated (Staley & Konopka 1985). Further in situ probing of river snow obtained from the Rhine River, the Oder River, and the Spree River also showed different amounts of this widespread beta-*Proteobacterium.* Moreover, these results are in

agreement with the study of Zwart et al. (1998a), who showed that this isolate is affiliated within a beta-sublineage, which comprises several other organisms from different aquatic habitats at various geographical sites in North America and Europe. Phylogenetic analysis of strain 21 revealed its closest relationship to *Matsuebacter chitosanotabidus* with a sequence difference smaller than 1% (99.1% similarity). *Matsuebacter chitosanotabidus* was collected and isolated from soil in Matsue, Japan and not from aquatic sources. This rises the question, whether strain 21, phylogenetically nearly identical with *Matsuebacter chitosanotabidus,* is originally an aquatic or soil bacterium? As described by Park et al. (1999) *Matsuebacter chitosanotabidus* produced a novel kind of chitosanase able to degrade deacetylated colloidal chitosan and glycol chitosan, both derivates of chitin. But the enzyme was not able to degrade chitin. Obviously, strain 21 is also unable to degrade chitin. Elbe batch cultures supplemented with chitin showed an immense decrease of algae and were dominated by a network of thick filamentous structures (Fig. 6B). But these cultures showed neither an increase of beta-*Proteobacteria* in general, nor within this group an increase of strain 21. Interestingly, strain 21 is also closely related to the new genus *Aquabacterium* gen. nov. (Kalmbach et al. 1999), including the strains *Aquabacterium citratiphilum* sp. nov., *Aquabacterium parvum* sp. nov. and *Aquabacterium commune* sp. nov. as type species. All three bacterial strains were isolated from biofilms of the Berlin drinking water system. Further characterization of biofilms throughout the distribution network demonstrated *A. parvum* and *A. commune* as dominant bacterial strains in groundwater and distribution system biofilms (Kalmbach et al. 1997b). Beside sequence similarity the cell morphology of strain 21 is also similar to *A. commune*. Individual cells of both strains were motile, Gram-negative rods with identical dimensions of 2 – 4 µm in length and 0.5 µm in diameter. They occurred as single cells, but tended to form cell aggregates. The cells contained dark inclusion bodies, which could be identified for *A. commune* as

polyalkanoate storage polymers. For an ecological understanding of the distribution of aquatic microorganisms, both isolates are important candidates for such examinations. Although closely related and similar in morphology, *Aquabacterium commune* and isolate 21 differ in their physiological abilities. The ecological niche concept, the responses of organisms to environmental variables, is useful in explaining these results. An ecological niche is the biological space within which an organism is active and reproduces in a given habitat (Hutchinson 1957). Although there are many possible multifactoral environmental aspects that may influence the survival of a given species, sometimes only a few environmental variables appear to significantly influence the niche space of aquatic microorganisms (Hutchinson 1978, Morowitz 1980). There are common ecological forces, which influence the whole subclass of beta-*Proteobacteria*, as for example salinity, which leads to the near exclusion of beta-*Proteobacteria* from open ocean environments. But based on our own results, within this phylum niche space must be regarded more difficile with respect to special clusters, groups and even strains.

Beside strain 21 the other five beta isolates could also be detected with the new developed probes. Cluster specific probes including the isolates and close relatives revealed all of them in different amounts as members of the microbial community of various riverine habitats. An interesting result is the detection of a great number of unique cells with probe Clu34kl specific for isolates strain 34, strain 11 and strain P10 and close relatives in young lotic microbial biofilms and their decrease during biofilm development. These findings are in agreement with the results of Manz et al. (1999), who also showed the dominance of beta-*Proteobacteria* in young lotic bacterial biofilms and their decrease in mature biofilms. Grossart & Ploug (2000), who investigated the phylogenetic composition of laboratory-made riverine aggregates also observed a striking decrease of beta-*Proteobacteria* during aggregate formation. All these results can be explained with the ecological niche concept. Obviously, the

conditions in mature biofilms or aggregates are less optimal for beta-*Proteobacteria* leading to their decrease and increase of a different phylum better adapted to the new conditions. In contrast to the other studies, the great advantage of the present study is the existence of the in situ detected cells as pure cultures of the isolated bacteria. Further investigations providing correlated informations about the phylogenetic identity and in situ distribution with physiological parameters will help to give answers to the complex questions of abundance of beta-*Proteobacteria* in freshwater environments. In this study phylogenetic analysis of the beta-*Proteobacteria* isolates revealed the majority of them as closely related to bacteria, capable of degrading unusual substrates. Strain P10 was closely related to *Hydrogenophaga palleronii* with the ability to grow aerobically with 4-aminobenzenesulfonate described by Blumel et al. (1998). Strain 14 was closest related to *Alcaligenes sp*. NKNTAU, a nitrate-reducing *Alcaligenes sp*. capable of utilizing taurine (Denger et al. 1997), and, as described above, the closest relative of strain 21 was *Matsuebacter chitosanotabidus* producing a novel kind of chitosanase able to degrade deacetylated colloidal chitosan and glycol chitosan. Together with the utilization of N-acetylglucosamine these results might give first hints of the enormous physiological potential within this subclass of *Proteobacteria* and a possible reason for their dominance in freshwater habitats. The performed Elbe batch culture experiments showed that N-acetylglucosamine favoured the growth of beta-*Proteobacteria* and within this phylum specifically the growth of strain 21 (Fig. 6E and F). In contrast, cells hybridizing with an *Aquabacterium commune* specific probe, presented only a minor part within the bacterial population. Although this enrichment culture situation cannot be compared to any changes observed in nature, the experiment gave useful informations about the strains (isolated from nature) and their physiological potential.

Most isolates obtained from R2A medium could be affiliated to lineages within the

gamma-*Proteobacteria* such as the genera *Aeromonas* and *Pseudomonas*. In contrast to this, gamma-*Proteobacteria* accounted in situ for only 20% of the bacterial river snow community. The preferential isolation of gamma-*Proteobacteria* on commonly used media is in good accordance with results of previous studies (Wagner et al. 1993, 1994). Moreover, there is obviously a widespread discrepancy between the in situ detection and cultivation of this phylogenetic group. In a study designed to explore the similarity of cultivated marine isolates with microorganisms detected using SSU rRNA sequence retrieval techniques Suzuki et al. (1997) showed, that the majority of the cultivated gamma-*Proteobacteria* had little similarity to the microorganisms, whose SSU rRNA sequence types were recovered from natural samples. In our study cultivation on R2A medium resulted in a rapid and extensive growth of gamma-*Proteobacteria,* overgrowing all other competing river snow bacteria. Obviously there is no causal relation between this observation and the physiological potential of the isolated Elbe river snow strains. The physiological profiles of the isolated gamma-*Proteobacteria* were similar to those of the isolated beta-*Proteobacteria.* Different affinities for nutrients might be a possible reason for cultivation efficiency. Phylogenetic analysis of the gamma-*Proteobacteria* isolates revealed most of them as members of the *Pseudomonadaceae* and *Aeromonadaceae*, both common inhabitants of aquatic sources. Whereas the *Aeromonadaceae* are known as potent pathogens (Farmer et al. 1992), *Pseudomonadaceae* are able to grow on a large variety of organic substrates. The application of the newly developed probe AERO1244, which is specific for the family *Aeromonadaceae*, showed their in situ relevance in river snow samples in spring and winter. Furthermore, in situ hybridization with probe AERO1244 detected a remarkable population shift within the gamma-*Proteobacteria*, which cannot be elucidated using a probe on the subclass level. This is in good agreement with results reported by Leff and coworkers (1998), who showed that differences on an

assemblage level can mask simultaneous changes occurring in populations of *Pseudomonas cepacia* in two North Eastern Ohio streams.

Although using R2A-agar plus casitone (specific for the isolation of *Cytophaga* and *Flavobacteria*) just one isolate (strain 8) was affiliated to this bacterial group. But in situ hybridization with the newly developed strain specific probe FLAVO1044 was able to detect this strain in Elbe river snow samples throughout the year. The next known relatives of this flavobacterial isolate are *Flavobacterium columnare* and *Flavobacterium johnsonae.* Both organisms belong to a group recently being detected by Kenzaka and colleagues (1998) at fairly high amounts in the rivers Minoh and Neya, which is an indication of the ubiquitous relevance of these bacteria in lotic ecosystems.

Planctomycetales could not be cultivated from the river snow community, although using a variety of specific media. However, rosette-like microcolonies, which are described as a typical feature for the as yet uncultured *Planctomyces bekefii* (Schlesner 1994), were regularly visualized in the river snow community by specific FISH. The *Planctomyces* phylum can be divided into three distantly related subdivisions: the *Chlamydiales*, the *Verrucomicrobiales*, and the true *Planctomyces* (Maidak et al. 1997, Ward-Rainey et al. 1995). Members of these subdivisions have been detected in all aquatic habitats but do not appear to be evenly distributed (Fuhrman et al. 1993, Gray & Herwig, 1996, Hiorns et al. 1997, Rath et al. 1998, Wise et al. 1997, Zwart et al. 1998b). In agreement with our own results all these observations were exclusively based on molecular techniques and not on cultivation efforts.

Gram-positive bacteria with high GC content of DNA could not be detected in situ, and the two bacterial isolates strain 37 and C2 constituted just a minor part of all cultivated bacteria. Nevertheless, the next known relative to strain 37 is *Mycobacterium* sp. 28, capable of degrading polycyclic aromatic hydrocarbons

(Gowindaswami et al. 1995). It was isolated from fossil-fuel contaminated environments. Because of their specific physiological potential *Mycobacteria* are important members of different bacterial communities.

Enrichment cultures of river snow under anaerobic conditions using mineral medium supplemented mainly with casamino acids resulted in the isolation of several Clostridia. Their phylogenetic affiliation shows that they were closely related to known Clostridia. As described by Rheinheimer (1985), clostridia are common and widely distributed inhabitants of aquatic ecosystems. Because of their major role in nature, the degradation of organic material to acids, alcohols, CO_2, H_2, and minerals, (Hippe et al. 1992), clostridia are important members of the river snow community. In fast running waters (as the Elbe River), clostridia may mainly exist in the form of endospores, whereas in oxygen depleted micro-niches vegetative cells of clostridia could be detected. The striking discrepancy of in situ detection with FISH and enrichment efficiency of clostridia might be due to endospore formation along with a presumably low metabolic potential of vegetative clostridia. Otherwise, the observation of large numbers of strongly fluorescent cells in anaerobic Elbe river snow enrichment cultures (Fig. 3G) using the newly developed probe CLOBU1022 demonstrates the principal presence of clostridia in the natural microbial community.

The isolation of strain 15.3 related to *Holdemania filiformis*, strain 15.5 related to *Anaerofilum agile* and strain PS5 related to *Anaeromusa acidaminophila* is a striking example for both: firstly, the enormous bacterial diversity of the river snow community and secondly, the success of the cultivation with a variety of specific media used in this study.

The four isolated SRB strains, all of them close relatives to *Desulfovibrio desulfuricans* belong to a phylogenetic cluster of sulfate reducing bacteria, which includes strain zt10e, and strain zt3l, isolated from the Berlin raw drinking water (Bade et al. 2000). Bade investigated the behaviour of sulfate reducing bacteria

under oligotrophic conditions and oxygen stress in coculture experiments with autochthonous drinking water species to elucidate the presumed protective function of aerobic bacteria on the survival of SRB. Possibly, the four Elbe river snow SRB isolates are good candidates for further investigations of SRB survival strategies in aerobic habitats.

Archaeal genes have been detected in relatively small proportion of pelagic marine microbiol communities (DeLong 1992, DeLong et al. 1994) but were comparatively more abundant in Antarctic surface waters (DeLong et al. 1994) and at deeper sampling sites in the open ocean (Fuhrman et al. 1993, DeLong et al. 1994, Giovannoni et al. 1995, Massana et al. 1997, Fuhrman & Ouverney 1998). In this study, methanogens were not detected in situ within the river snow community. However, the dominance of *Methanosarcinaceae* in an enrichment culture of the Elbe river snow (Fig. 3D) pointed out their principal presence, but putatively inactive metabolic state or very low cell numbers in the river snow community.

4.4. *Advantages of the polyphasic aproach*

Molecular biological approaches have revolutionized microbial ecology during the last decade and provided new insights into the structures of aquatic bacterial communities (Fuhrman et al. 1992, Giovannoni et al. 1990, Øvreås et al. 1997, Schmidt et al. 1991). One molecule in particular, the small subunit ribosomal RNA (SSU rRNA) has been extensively used as an evolutionary marker for the identification of microbial species (Woese 1994). By direct DNA extraction molecular diversity can be investigated by a wide range of molecular methods. SSU rRNA sequence retrieval techniques such as PCR amplification and genomic clone library construction (Schleper et al. 1997, Stein et al. 1996) appear along with in situ methods designed to detect and identify nucleotide sequences in individual microbial

cells (FISH). In the present study phase contrast microscopy and molecular methods were successful in discovering Planctomyces as members of the Elbe river snow community whereas an extensive cultivation approach failed to isolate this phylum. But nevertheless, the information content of molecular biological studies is limited. Firstly, there are significant methodological differences, such as the use of different cell lysis and nucleic acid extraction techniques, the use of different primers and conditions as well as various cloning and probe hybridization conditions. Secondly, molecular techniques can provide a distorted view of microbial community composition by preferentially amplifying certain sequence types (Farrelly et al. 1995, Reysenbach et al. 1992, Suzuki & Giovannoni 1996), creating artificial sequence diversity (Amann et al. 1996), and imparting bias during clone library construction (Rainey et al. 1994).

In the present study FISH was unable to detect Clostridia and methanogens in the bacterial river snow community obviously due to their low metabolic activity under the existing oxygen pressure. Cultivation under anaerobic conditions revealed *Methanosarcina* and a variety of different Clostridia and as members of the microbial aggregate associated community. But one should keep in mind that just one single spore or cell is enough for cultivation. Opponents of cultivation-based techniques for the observation of microbial community composition criticize the minimal amount (less than 1%) of culturable bacteria from environmental samples described as “the great plate count anomaly” by Staley & Konopka (1985). Furthermore they criticize the little similarity between the isolated bacteria and the SSU rRNA sequence types recovered from the same natural sample (Suzuki et al. 1997). Based on our own results we must reject this thesis. The use of unconventional low nutrient media close to natural conditions led to the cultivation of a variety of in situ relevant and ubiquitous bacteria (for example the isolated beta strains). Furthermore the remarkable kind of cell organisation of strain F8, isolated on an oligotrophic medium

from river snow of the South Saskatchewan river (for detailed information see discussion below), could not be revealed by its DNA sequence data but was discovered by microscopical investigations of the cultivated strain. Therefore, we believe that the polyphasic approach of combined FISH and cultivation applied in this study led not only to a comprehensive description of the bacterial river snow community but will also contribute to a better understanding of the correlation of phylogenetic diversity with the influences of ecological forces in lotic systems. Nevertheless, if we want to understand the control of the diversity of microbial communities from the bottom up, studies on the biochemistry of the same samples are necessary and new experimental approaches are needed. For a comprehensive understanding of the microcosmos “lotic aggregates” the present study should be connected with other studies, which examined different aspects of this habitat. Zimmermann (1997) and Wörner et al. (2000) for example investigated the relation of protozoans and metazoans to aggregates (Zimmermann 1997, Wörner et al. 2000). The succession of protists on laboratory-made newly formed aggregates indicated the existence of a microbial food web within the aggregates based on bacterial production. Grossart combined radiotracer with microsensor techniques to measure bacterial production and growth efficiencies on riverine aggregates (Grossart & Ploug 2000). These results demonstrated that bacterial production and respiration on aggregates were dependent on the bacterial community and the substrate composition of aggregates. In situ cell and glycoconjugate distribution in native river snow (Neu 2000) indicated that the chemical heterogeneity of river snow may be significant for sorption and transport of nutrients and contaminants in lotic aquatic environments.

4.5. *Detection of non cellular components with the use of lectins, their abilities and limitations*

Lectins have been known for more than a century. They were regarded as bi- or multivalent carbohydrate-binding proteins. Allmost all microorganisms express surface-exposed carbohydrates. The carbohydrates may be covalently bound, as in glycosylated teichoic acids to peptidoglucan, or non-covalently bound, as in capsular polysaccarides. Every surface–exposed carbohydrate is a potential lectin-reactive site. The specificity of the recognition process is determined by stereochemical principles. In addition, the carbohydrates interact with lectins via hydrogen bonds, metal coordination, van der Waals, and hydrophobic interactions (Elgavish & Shaanan 1997). The ability of lectins to complex with microbial glycoconjugates has made it possible to employ the proteins as probes and sorbents for whole cells and numerous cellular constitutents and metabolites. Microbial receptors for lectins consist of several unique chemical structures. Although lectins are carbohydrate-recognizing proteins they may have bi-functional properties with a lectin-carbohydrate and additionally a lectin-protein-binding site (Sharon & Lis 1989). Fluorescently labelled lectins are one of the most promising tools for probing the EPS matrix in biofilm systems (Neu & Lawrence 1999a) and lotic microbial aggregates (Neu 2000). Whereas nucleic acid specific stains and FISH were used to reveal the distribution of single microorganisms or microcolonies immobilized in the non-visible polymeric matrix, fluorescently labelled lectins can demonstrate the space in between the individual microorganisms. This “empty” space may be represented by EPS of microbial and algal origin as well as humic substances and may have a crucial role in the interaction and transport of nutrients and contaminants in lotic systems. Nevertheless, in general lectin-specificity should be discussed in a critical way. Firstly, the majority of the isolated and characterized lectins are originating from

eucaryotic organisms, including seeds, plants, fungi, sponges, molluscs, body fluids of invertebrates and lower vertebrates and from mammalien cell membranes (Gold & Balding 1975). The precise pysiological role of lectins in nature is still unknown and variable. Recently Rickard et al. (2000) showed, that coaggregation between aquatic bacteria was mediated by specific-growth-phase dependent lectin-saccharide interactions. Secondly, lectin's known sugar specificity is restricted so far to common carbohydrates as given by the suppliers data sheets. Additionally some lectins have not only an affinity for a monosaccharide but for di-, tri-, and even oligosaccharides. In natural samples unknown glycoconjugates or carbohydrate-combinations might exist, which also bind to the offered lectins. We cannot always be sure that binding is originating only from the known sugars but also from lectin-protein interactions. Nevertheless, control experiments in this study clearly showed the inhibition effect of D-glucuronic acid and N-acetylneuraminic acid on the binding of *Limulus polyphemus* FITC-lectin to river snow. This is in agreement with other reports. Neu (2000) demonstrated the methyl-α-D-mannopyranoside inhibition effect on the binding of the lectin from *Canavalia ensiformis* and Michael & Smith (1995) used the identical lectin and carbohydrate combination in control experiments with environmental biofilms. Therefore, LBA applied in this study visualized different kinds of binding although the nature of the binding remains in some cases unknown.

4.6. *Structural analysis from the level of overall distribution up to cell envelopes*

Using a variety of lectins, the new method visualized the distribution of different glycoconjugates in lotic microbial aggregates at several orders of magnitude. For extraction of three types of information the confocal laser scanning microscope was run in the three channel mode. This approach allowed the simultanous collection of three signals in the green (FITC), red (CY3) and far red (autofluorescence) channels

(Lawrence et al. 1998). Earlier attempts of CLSM to study aquatic aggregates (Droppo 1996, Holloway and Cowan 1997, Liss1996) used samples after embedding in agarose, which may interfere wit the multiple application of lectins (Droppo et al. 1996, Liss et al. 1996). In this study fluorescently-labelled lectins were used to probe both fully hydrated living river snow samples analysed by CLSM in the reflection mode and samples after formaldehyde fixation (a necessary step for FISH) analysed in the multi channel mode. *Triticum vulgaris* lectin stained major parts of the whole floc structure. Similar results could be observed in river biofilms (Lawrence et al. 1998, Neu & Lawrence 1997), but did not correspond with the results of lotic aggregates obtained from other rivers, e.g. the South Saskatchewan River, Canada. Whereas *Triticum vulgaris* lectin stained only minor parts of these lotic aggregates, lectin obtained from *Glycine max*, which is characterized by the affinity for N-acetyl-D-galactosamine, stained major parts of these river snow aggregates (data not shown). The majority of the hybridized bacteria were located at the surface of the aggregates. These results are in agreement with lake snow aggregate formation and bacterial colonization in lake Kinneret, Israel. Grossart et al. (1998) observed high numbers of colonizing bacteria on the surface of the aggregates which were covered by a thick layer of mucopolysaccharides.

Distinct EPS compartments within the river snow matrix became visible after simultaneous nucleic acid staining with DAPI and staining with lectin for example from *Pseudomonas aeruginosa* (Fig. 9E). Obviously, the new method allowed the distinction of morphologically uniform bacteria by their EPS components. Although a large number of morphologically uniform filamentous bacteria could be observed, the *Pseudomonas aeruginosa* lectin stained only the cell-envelopes along a bundle of certain filamentous bacteria. Consequently the aim of the new method should be the characterization and affiliation of EPS to a certain producer even on the level of single bacteria. Detailed studies of defined cocultures under lab conditions as well as

more in situ approaches are necessary.

Simultaneous staining with probe BETA42a specific for the beta subclass of *Proteobacteria* (Manz et al. 1992) and FITC-labelled lectin from *Arachis hypogaea* visualized a chain of rod shaped bacteria embedded in a polysaccharide matrix (Fig. 9F). These observations demonstrate that the new method had not only the ability to distinguish clearly between the bacterial cell and the surrounded material. Moreover the technique allowed the affiliation of single cells to certain bacterial phyla and the characterization of the surrounding slime layer. Based on their striking morphology the beta-*Proteobacteria* shown in Fig. 9F might belong to the sheathed bacteria which represent a minor lineage among this subclass. They are common inhabitants of running fresh waters, but also occur in polluted aquatic habitats including sewage or waste water from paper, potato, dairy and other agricultural industries (Mulder & Deinema 1992). As described by Gaudy & Wolfe (1962) the slime polysaccharide e.g. of the beta-*Proteobacteria Sphaerotilus-Leptothrix* group is composed of the carbohydrates fucose, galactose, glucose and glucuronic acid. This composition is clearly different from the sheath material, which consists of a complex protein-polysaccharide-lipid structure without peptidoglycan (Romano & Peloquin 1963). The presence of both sheath and slime layer has significant ecological and nutritional consequences for the organisms as described by Mulder & Deinema (1992), including the ability to attach to solid surfaces and the protection and shelter against predation and virus infection (Venosa 1975).

Lectin from *Pseudomonas aeruginosa* was able to stain the radial shaped polysaccharide matrix which surrounded a special green algae (Fig. 9G). The combination of the same lectin and probe EUB338 visualized small rod shaped bacteria in the thick slime layer of the green algae *Scenedesmus spp*. (Fig. 9H). These findings might help to analyse the various bacteria-phytoplankton interactions in aquatic systems. They range from symbiosis to parasitism and are highly variable

in space and time. Grossart et al. (1999) studied the growth of three marine diatoms in axenic culture incubated under controlled lab conditions with single bacterial isolates. They showed, that algal exopolymer production had a significant influence on bacterial colonization and that bacterial production and exoenzyme activity could lead to a modification of exopolymeric material.

An interesting finding is the visualization of certain bacterial cells and envelopes after double staining with probe EUB338 and lectin from *Phaseolus vulgaris* (Fig. 9I). Whereas a variety of different morphological cells hybridized with the probe, only an assemblage of uniform thick rod shaped bacteria showed a lectin conferred signal located at the cell surfaces. As given by the material data sheet of the supplier *Phaseolus vulgaris* lectin has no specific affinity for any well known sugar. The cell-envelopes of the uniform bacteria are obviously composed of a certain substance which is able to bind to the lectin, but the nature of this substance is unknown. Nevertheless, the results underline both, (i) the potential of the lectins to differentiate between various bacteria and (ii) the potential of lectins to analyse spatial structures.

The new combined technique presented in this study is fast and easy to perform. Different lectins stained distinct glycoconjugates originating from different eucaryotic and procaryotic microorganisms. Lectin from *Limulus polyphemus* even stained cell-envelopes of certain bacterial river snow isolates. The in situ structural analysis of extracellular polymeric substances in “interfacial microbial communities” completes the polyphasic approach of this study of molecular biological and cultivation approaches. To our knowledge up to now no other method combines information of the phylogenetic identification on the cellular level obtained by FISH with the analysis of the spatial distribution of the extracellular polymeric substances detected by LBA. This combined approach will be a valuable tool for investigations concerned with microbial and glycoconjugate identity not only in natural, but also in artificial (e.g. the biodegradation of xenobiotics) and medical habitats (e.g. bacterial infections of

endoprotheses).

4.7. *Filaments of strain F8: detection and nature*

The results reported here indicate that filament production by strain F8 was dependent on specific nutrient conditions. Strain F8 failed to grow or grew weekly in rich media (NB and TSA), indicating that these are not the conditions it is adapted to in its natural aquatic environment. Growth in R2A medium initially designed for heterotrophic organisms in treated potable water was excellent but never showed the formation of filaments. Lower nutrient media (FBM, RRW) resulted in poorer bacterial growth but increasing filament formation. This might indicate, that filament formation is an induced behaviour and a response to low or decreasing nutrient conditions in the natural environment. The observation of filament production in an initially filament free F8 culture grown in R2A medium after transferring to FBM medium underlines and is in keeping with this thesis.

Although a large number of stains was used for the characterization of the bacterial filaments clear conclusions regarding the chemical composition of the filaments are still missing. The positive staining result with tannic acid, specific for flagella protein, might indicate a possible protein nature of the filaments, but disagreed with the results of the two protein stains SYPRORed and SYPROOrange. Both of them gave no signals with the filaments. A postulated polysaccharide nature of the filaments could be rejected with respect to known polysaccharides. None of the 17 lectins used in this study, which may specifically bind to a wide range of different mono- di- tri- and polysaccharides, were able to stain the filaments. The excellent visualization of the filaments with different DNA-specific SYTO-dyes and SYTOXGreen scarcerly implies the presence of nucleic acides in the filaments. But, the failure of DAPI to positively stain these structures may indicate a non-specific

staining behaviour of the dyes. The staining results indicate that filaments are either composed of an up to now unknown material or probably a mixture of several substances. The TEM images (Fig. 11D and E) reveal the secretion of a membrane surrounded material from the cell surfaces. Close to cell surface the material is of small round shape with the ability to elongate and to connect with other round shaped structures to form filamentous structures. Polysaccharide, protein, lipopolysaccharide or even DNA nature of the material is thinkable. More detailed chemical analysis of purified filament materials are strongly required to resolve this issue.

4.8. *Cell organization in the bacterial kingdom*

The present study described the formation of unusual filaments of the bacterial strain F8. The process can be considered as a kind of cell organization and is a striking example of microbial cell-cell interactions. A bacterial strain developed from single cells into a structure composed of many cells (possibly the beginning of a multicellular state). Individual cells initiated the process with the production and accumulation of a material which is able to elongate into filaments. Through the production of these filaments one cell connected with other cells and a complex filamentous network established.

In the bacterial kingdom there are a variety of cell organization forms including extracellular polymeric substances. Cell organization ranges from simple stadium to complex life cycles. The sheathed bacteria for example are common inhabitants of running fresh waters (Mulder & Deinema 1992). A sheat is a hollow structure, composed of a complex protein-polysaccharide-lipid structure without peptidoglycan (Romano & Peloquin 1963) that surrounds a chain of cells. It is often closely fitting but is not in intimate contact with the cells. This can be concluded from the ability of

the cells to move out of the sheats appearing as free swimming single cells, so called swarmers, provided with polar flagella. As described by Gaudy & Wolfe (1962) the sheats of several *Sphaerotilus* and *Leptothrix* species are surrounded by an additional slime layer. In aggreement with the filament formation of strain F8, the formation of a sheat is affected by the composition of the culture medium. In media rich in organic nutrients *Sphaerotilus natans* forms no sheats. The presence of both sheath and slime layer has significant ecological and nutritional consequences for the organisms as described by Mulder & Deinema (1992), including the ability to attach to solid surfaces and the protection and shelter against predation and virus infection (Venosa 1975). The *Planctomycetales* are another example for a procaryotic multicellular state. These microcolonies consist of large numbers of spherical cells, typically linked via a gelatinous material at the distal tips by stalks (Staley 1968, Staley et al. 1992). Although up to now not cultured in this complex form, molecular biological studies revealed their widepread occurence in aquatic habitats (Neef et al. 1998). The photoautotrophic Cyanobacteria, common inhabitants of a variety of aquatic environments, use light as source of energy, water as electron donor and CO_2 as carbon source (Stanier & Cohen-Bazire 1977). Living in microbial mats they suffer from self-shading caused by the extremely densely-packed organisms. Hence, motility is of great importance to these organisms, enabling them to position themselves in optimal light. Many cyanobacteria excrete extracellular polymeric substances that are mainly composed of polysaccharides (Decho 1990). The material is loosely associated with the cell or is freely present in the medium and is known as mucilage or slime. The hypothesis of gliding motility by self-propulsion as a result of the secretion of slime finds it origin in some ultrastructural cell wall pores of a number of cyanobacteria. These pores were assumed to be the sites from which slime was secreted. However Halfen & Castenholz (1971) have found microfibrills in the outer cell layers of *Oscillatoria princeps*. They suggested that these microfibrills

were contractile and that these would generate surface undulations that could propel the cyanobacterium on a solid surface. Both hypothesis fit partly with the behaviour of strain F8. The first step of filament formation, the accumulation of a material around the cells, might be the result of material excretion of each bacterial cell as shown on the TEM images. The positive staining result of the filaments with tannic acid, specific for flagella protein, might allow the comparison with the cyanobacterial microfibrills. In the early sixties Perfiliev (1954, 1960), Perfiliev & Gabe (1961) and Aristovskaja (1961) described a group of highly organized rod shaped bacteria. They were able to form network-like microcolonies of cells and extracellular polymeric substances and able to move like a multicellular organismn. Häusler (1982) summerized this phylogenetic order of bacteria as *Cyclobacteriales* with the genus *Cyclobacter*, *Teratobacter*, *Desmobacter*, *Streptobacter*, *Trigonobacter* and *Dictyobacter*. Whereas *Cyclobacter* and *Teratobacter* produced filaments in form of a simple loop, *Desmobacter, Streptobacter* and *Trigonobacter* formed a spiderweb-like complex network for preying single bacteria. *Dictyobacter* was even able to form a bag-like net to capture single bacteria. All these bacteria were just detected by microscopical examination in a variety of freshwater lakes and ponds. Because up to now none of them was isolated in pure culture, their presence is discussed in a critical way. Nevertheless strain F8 showed a striking similarity in its network forming behaviour to this bacterial order, although the function of this structure remains unclear. Strain F8 has the advantage of being isolated and available for detailed investigations. Other bacteria with the property of ”social behaviour” like the Myxobacteria manifest cell-cell interaction in their fruiting body development (Rosenberg 1984). Whereas these gram-negative soil inhabitants occupy an exceptional position within the bacteria kingdom, strain F8 is a common rod shaped deep branching gamma-*Proteobacterium*. This is also an interesting finding from the evolutionary perspective. Cell to cell interactions are described for simple eucaryotic

organisms such as *Chlamydomonas reinhardtii* a unicellular biflagellate green algae (Goodenough 1977) and the amoebae *Dictyostelium* (Schaap 1986). The dictyostelids represent an interesting group of species, which are hovering on the borderline between uni- and multicellularity. The developement of these organisms is discussed as demonstrating how, during the course of evolution, the initial step to multicellularity may have been made. Strain F8 as a procaryotic organism is obviously also capable of cell organization. This might indicate that complex cell organization started much earlier in the course of evolution that has been considered to date.

4.9. *Possible advantages of cell organization*

As described by Dworkin (1991) there is obviously no common theme of microbial cell-cell interactions. They are different strategies that microbes have evolved as alternatives to their fundamentally unicellular nature. They can be divided in (i) mating interactions, (ii) developmental interactions, (iii) ecological/colonization interactions and (iv) predator-prey interactions. The network forming behaviour of strain F8 might be considered as ecological/colonization or predator-prey interaction. In the course of evolution the formation of networks seems to be a successful strategy for food assimilation for a variety of organisms living in aquatic habitats particularly in running waters. As organic matter in streams and rivers is transported downstream *Hydropsychidae* larvae are able to form complex networks optimal adapted to the direction of flow (Fey 1992). Larvae of *Philopotamidae* also form networks in which they live on netted diatoms and organic detritus (Elliot 1981). Perhaps the formation of a network also enables strain F8 to assimilate nutrients. As a member of a microbial aquatic community in floating aggregates, in which competition may strongly influence microbial species, this behaviour may offer strain

F8 a survival strategy in resource competition.

4.10. *The role of signal molecules*

It is easy to imagine that cell communication requires signal molecules. Known cell to cell signaling systems in the bacteria kingdom are acylated homoserine lactones (AHLs) which are chemical signals that mediate population density-dependent (quorum-sensing) gene expression in numerous gram-negative bacteria (Geiger 1994). These autoinducer signal molecules control such diverse processes as bioluminescence by *Vibrio fischeri* or *Vibrio harveyi* (Cao & Meighen 1989, 1993, Eberhard et al. 1981, Fuqua et al. 1994), the virulence of the plant pathogen *Erwinia carotovora* (Pirhonen et al. 1993) and of the opportunistic human pathogen *Pseudomonas aeruginosa* (Gambello & Iglewski 1991, Ochsner et al. 1994, Passador et al. 1993, Pearson et al. 1994) as well as the conjugative transfer of the tumor-inducing plasmid of *Agrobacterium tumefaciens* (Winans 1993, Zhang et al. 1993). Recently AHLs have also been detected in naturally occuring biofilms (McLean et al. 1997). First results (not presented in this study) reveal strain F8 as an AHL producer. In agreement with filament formation AHL production by strain F8 seems to be correlated with media conditions.

The use of the oligotrophic medium FBM for cultivation and CLSM analysis with fluorescent labeled dyes led to the detection of the stream bacterium F8. Strain F8 is an eubacterium capable of a remarkable process of cell organization along a self-produced filamentous network. The observed behaviour has impications for understanding strategies of surface colonization, floc formation and resource competition in natural environments. The evolutionary aspect of procaryotic cell organization as well as the technical application of the bacterial produced filaments

should be of great microbiological interest. Detailed studies dealing with the nature of the movement, the filaments nature, signal molecules and biofilm developement on special surfaces are in progress.

4. 11. *Outlook*

The goal of ecological studies should be first to describe natural phenomena, then to explain, predict, and ultimately control natural processes (Begon et al. 1990). The present study gives a detailed proximal and ultimate description of the bacterial Elbe river snow community. Proximal explanations describe the ecology of organisms in terms of their phenotypic adaptations to environmental variables. Ultimate explanations are based on studies of genotype divergence and provide an evolutionary history of adaptive change (Mayr 1997). But we must, however move beyond descriptive studies and begin to perform experimental manipulations of environmental variables in order to predict the effects of environmental change on aquatic microbial communities. Because of their in situ relevance and dominance the isolated beta-*Proteobacteria,* especially strain 21, are suitable candidates for such investigations. The interesting results of first experimental manipulations as the Elbe batch culture experiments with different unusual nutrients should be further investigated. They might give explanations on the complex question of the dominance of beta-*Proteobacteria* in freshwater environments. Due to its easy performance, the new combined technique of FISH and LBA should become a routine-method to check cellular and noncellular components of microbial communities. Moreover detailed studies of the unusual behaviour of strain F8 and of filaments nature are strongly desired. Via molecular biological methods it should be examined whether strain F8 is an ubiquitous and in situ relevant member of microbial communities of different aquatic environments. Coculture experiments should reveal

if the behaviour provides any possible advantages in competition with other bacteria. Beside these “artificial” experiments more in situ approaches dealing with all aspects of aquatic ecology are desired. Such a new approach could be the incorporation of specifically labelled substrates into biomarkers as demonstrated recently for ^{13}C-labelled acetate into fatty acids of natural sediment communities (Boschker et al. 1998).

Therefore, a combination of molecular biological techniques, cultivation techniques, structural analysis and organic chemical approaches might help unravel the major driving forces behind the seasonal successions of microbial communities and the competition among its individual bacterial components.

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