

# Low Intraspecific Diversity in a *Polynucleobacter* Subcluster Population Numerically Dominating Bacterioplankton of a Freshwater Pond

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**Cultivation-dependent and -independent methods were combined to investigate the microdiversity of a *Polynucleobacter* subcluster population (Betaproteobacteria) numerically dominating the bacterioplankton of a small, humic freshwater pond. Complete coverage of the population by cultivation allowed the analysis of microdiversity beyond the phylogenetic resolution of ribosomal markers. Fluorescent in situ hybridization with two probes specific for the narrow subcluster C (PnecC bacteria) of the *Polynucleobacter* cluster revealed that this population contributed up to 60% to the total number of bacterioplankton cells. Microdiversity was investigated for a date at which the highest relative numbers of PnecC were observed. A clone library of fragments of the ribosomal operon (16S rRNA genes, complete 16S-23S internal transcribed spacer 1 [ITS1], partial 23S rRNA genes) amplified with universal bacterial primers was constructed. The library was stepwise screened for fragments from PnecC bacteria and for different ITS genotypes of PnecC bacteria. The isolated PnecC strains were characterized by sequencing of the 16S rRNA genes and the ITS1. Both the clone library and the established culture collection contained only the same three ITS genotypes, and one of them contributed 46% to the entire number of clones. Genomic fingerprinting of the isolates with several methods always resulted in the detection of only one fingerprint per ITS genotype. We conclude that a *Polynucleobacter* population with an extremely low intraspecific diversity and an uneven structure numerically dominated the bacterioplankton community in the investigated habitat. This low intraspecific diversity is in strong contrast to the high intraspecific diversities found in marine bacterial populations.**

Microdiversity of prokaryotes, i.e., the genetic diversity within species-like (>97% similarity of 16S rRNA genes) phylogenetic groups, receives increasing attention in microbial ecology (1, 5, 18, 19, 24, 28); however, the ecological significance of this diversity is still unknown. The coexistence of different bacterial genotypes belonging to the same species-like phylogenetic group is well documented for marine (1, 18, 19, 35, 45) and freshwater habitats (8, 9, 10, 11, 27, 30, 43). Recently, Acinas et al. (1) demonstrated by the construction and analysis of 16S rRNA clone libraries that a coastal bacterioplankton community contained a very high diversity of ribotypes, the vast majority of which fell into phylogenetically microdiverse sequence clusters (<1% divergent 16S rRNA sequences). Similarly, extensive microdiversities were also observed in populations of sulfate-reducing bacteria inhabiting a salt marsh (29) and in a *Vibrio splendidus* population from coastal bacterioplankton (42). The *V. splendidus* population consisted of at least a thousand distinct genotypes, which demonstrated a high variability in genome size and allelic composition (42).

The major aim of the study presented here was the investigation of the intraspecific structure (microdiversity) of a bacterial population occurring in freshwater bacterioplankton with high numbers. Specifically, we wanted to reveal (i) if populations of freshwater bacteria share with marine popula-

tions the characteristic of a high intraspecific diversity and (ii) if a population contributing >50% of the total numbers of bacterioplankton cells possesses a high genotypic diversity, which could potentially indicate a high intraspecific ecological diversity within the population.

A *Polynucleobacter* subcluster C (PnecC) (23) population (operationally defined as a narrow ribotype cluster), which temporarily dominated the bacterioplankton of a small dystrophic freshwater habitat, was selected for the study. The monophyletic PnecC cluster is characterized by a high minimum sequence similarity of 98.5% (23) and resembles in this feature the microdiverse clusters of marine bacteria (1, 42). Sequences affiliated with PnecC were previously obtained from endosymbionts of aquatic ciliates (40, 44), from many freshwater habitats investigated with culture-independent methods (11, 21, 27, 50), and from strains isolated from several freshwater habitats (23). Reports on the detection of PnecC bacteria in soil or marine systems are lacking. Due to the differences in origin of the sequences, it was previously speculated that the species-like PnecC cluster contains ecologically diverse strains, i.e., obligately endosymbiotic, as well as free-living, strains (23); however, evidence for the fundamental differences in lifestyle between PnecC strains observed in the cytoplasm of benthic ciliates (19, 44) and strains isolated from the pelagic zones of several freshwater habitats (23) is still lacking. Furthermore, it is not known if the many PnecC sequences retrieved by culture-independent methods from freshwater habitats, as well as the isolated strains, originate from cells contained in ciliate cells or from free-living cells. This knowledge is important for the interpretation of the ecological role of PnecC bacteria, because

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TABLE 1. Newly designed FISH probes specific for *Polynucleobacter* bacteria<sup>a</sup>

Probe	Sequence	Length (nt)	Target	Position <sup>b</sup>	Formamide concn <sup>c</sup> (%)	Specificity	No. of unspecific matches <sup>d</sup>
PnecABD-445	5'-GAG CTG CTG TTT CTT CCC-3'	18	16S rRNA	445-463	35	Subclusters A, B, and D	0
PnecC-16S-445	5'-GAG CCG GTG TTT CTT CCC-3'	18	16S rRNA	445-463	35	Subcluster C (PnecC)	1 <sup>f</sup>
PnecC-23S-166	5'-GTT CGC TTC TCA TAC CCT-3'	18	23S rRNA	166-182	35	Subcluster C (PnecC)	0
PnecD1-181	5'-TTT CCC CCT AAG GGA TT-3'	17	16S rRNA	181-197	15	Part of subcluster D <sup>e</sup>	1 <sup>g</sup>
PnecD2-181	5'-TTT CCC CCT TAG GGA TT-3'	17	16S rRNA	181-197	15	Other part of subcluster D <sup>e</sup>	3 <sup>h</sup>

<sup>a</sup> Information on the division of the *Polynucleobacter* cluster in four subclusters (A to D) can be found elsewhere (23).

<sup>b</sup> *E. coli* numbering.

<sup>c</sup> Concentration in hybridization buffer.

<sup>d</sup> Number of known sequences which match the probes but do not belong to the target group.

<sup>e</sup> Sequences of isolate MWH-CaK1 (AJ550667) and clone 27 (AF361194) are not targeted by these probes (both belong to subcluster D).

<sup>f</sup> Uncultured *Cytophagales* bacterium clone LiUU-1-2B (AF550604). Sequence was published by Eilers et al. (13).

<sup>g</sup> Environmental sequence (AY592152) was obtained by Heijs et al. (unpublished data) from an anaerobic marine site.

<sup>h</sup> Two *Beggiatoa* sequences (AF110273, AF110274) and one alphaproteobacterial sequence from a hot spring (AF445716).

free-living and endosymbiotic stages have different ecological functions and participate differently in carbon and nutrient fluxes of ecosystems.

The microdiversity of the selected PnecC population was investigated by construction and analysis of a clone library containing fragments of the bacterial ribosomal operons. In order to characterize the microdiversity of the population beyond the phylogenetic resolution of ribosomal markers, a culture collection of PnecC strains was established and analyzed by way of the sequencing of ribosomal markers and genomic fingerprinting by five different methods. The combination of culture-independent and cultivation methods provided deep insights into the genotypic structure of a bacterial population numerically dominating a freshwater community.

#### MATERIALS AND METHODS

**Study site.** The *Polynucleobacter* subcluster C (PnecC) population in a permanent, small (surface area, ~150 m<sup>2</sup>), shallow (maximum depth, 1.3 m), humic pond called Kleine Lacke was investigated. This fishless pond is located in the Austrian Alps (13°18'N, 47°44'E) near Salzburg at an altitude of 1,300 m above sea level. The pond contains acidic water with a brown water color. The entire shore of the pond is formed by "schwimmoor" (i.e., floating peat moss [*Sphagnum*]-dominated vegetation). The pond is lacking a permanent surface inflow and possesses an almost permanent outflow with low water charge. The surrounding terrestrial area is approximately equally covered by grass, blueberries, ferns, and coniferous trees. The area is usually used as a pasture, stocked with a small number of cattle and horses, in the period from June to mid-September. In the investigation period, the pond was permanently ice and snow covered from December 2003 to early May 2004. In February, the ice cover was ca. 0.5 m thick and consisted of several layers of different consistencies.

**Sampling.** The pond was repeatedly sampled over a period of 13 months (September 2003 to October 2004). Oxygen concentration, pH, conductivity, and water temperature were measured on location. For the determination of microbiological data, water samples were transported to the lab and immediately processed upon arrival. Transportation times were usually less than 1 h.

**Determination of total bacterial cell numbers.** Water samples were fixed with formaldehyde (2% final concentration), stained with 4',6'-diamidino-2-phenylindole (DAPI; Sigma), and filtered onto black 0.2- $\mu$ m-pore-size Nuclepore filters (Millipore). Total bacterial cell numbers were determined with an epifluorescence microscope (Zeiss Axioplan) under UV excitation at a magnification of  $\times 1,250$ . Calculated cell numbers are based on at least 10 counted microscopic fields.

**Development of probes for FISH.** A set of five probes specific for subclusters or for groups of subclusters of the *Polynucleobacter* cluster (23) was designed by using the ARB software package (32). Additional theoretical evaluation of the specificity of probes by the submission of probe sequences to BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) resulted in a few unspecific matches (Table 1); however, the prokaryotes potentially matching the probes are with one exception not known from the pelagic zone of freshwater habitats. The results obtained with

the only probe (PnecC 16S-445) unspecifically matching a planktonic freshwater bacterium were checked by a second probe specific for the same phylogenetic group. The determination of stringent conditions for fluorescent in situ hybridization (FISH) was performed by using paraformaldehyde-fixed cells of target and nontarget strains. These experiments were performed with cultures of *Polynucleobacter* strains, as well as with strains not affiliated with this cluster. Fluorescence intensities of hybridized nontarget and target cells were analyzed in experiments with a series of increasing concentrations of formamide in the hybridization buffer. Under stringent hybridization conditions, the subcluster-specific probes did not hybridize with members of the other subclusters. The sequences of the five developed probes and their stringent hybridization conditions are provided in Table 1.

**Analysis of samples by FISH.** Samples (20 ml) were fixed with paraformaldehyde solution (2% [wt/vol] final concentration) at room temperature for 2 h. After fixation, samples were filtered onto 0.2- $\mu$ m-pore-size Isopore membrane filters (47 mm; Millipore), rinsed with phosphate-buffered saline buffer and sterile Milli-Q water, dried at room temperature, and stored at -20°C. Whole-cell in situ hybridizations of sections from the polycarbonate filters were performed with the five newly designed oligonucleotide probes, as well as with probes BET2-870 (*Polynucleobacter* cluster and a few related sequences outside the cluster, 16S rRNA targeted) (7), BET42a (*Betaproteobacteria*, 23S rRNA targeted) (34), GAM42a (*Gammaproteobacteria*, 23S rRNA targeted) (34), ALF968 (*Alphaproteobacteria*, 16S rRNA targeted) (37), CF319a (many *Bacteroidetes*, 16S rRNA targeted) (33), and EUB338 (most bacteria, 16S rRNA targeted) (3). All probes were obtained as Cy3-monolabeled probes (Thermo-Hybaid, Ulm, Germany). Hybridization and analysis were performed according to the protocol by Alfreider et al. (2).

**Isolation and cultivation of PnecC strains.** For the isolation of PnecC strains from a water sample taken on 15 October 2003, the acclimatization method (25) was used in two variations. The first variant, the filtration-acclimatization method, uses filtration through 0.2- $\mu$ m-pore-size filters for the enrichment of *Polynucleobacter* bacteria, as well as for the exclusion of many bacteria that are able to overgrow the target bacteria. The second variant, the novel dilution-acclimatization method (DAM), uses dilution steps for separation of the most abundant bacteria from the less abundant ones. In general, the cultivation experiments were performed as described previously (25). The only differences were the dilution steps in DAM, as well as the use of additional media. Three different dilutions ( $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$ ) were incubated in the different DAM experiments. Three different media were used for the initial cultivation steps of filtration-acclimatization method and DAM experiments. These media were the artificial inorganic IBM medium (25) enriched with 0.1% NSY medium (25) and the 0.2- $\mu$ m-filtered water from the investigated habitat, sterilized either by autoclaving or by microwave treatment (39). Cultures were screened by FISH for the identification of PnecC-positive cultures. Only PnecC strains were further processed and analyzed.

**Phylogenetic analysis of the isolated PnecC strains.** Partial (all strains) and almost complete (12 strains) sequencing of the 16S rRNA gene was performed as described previously (23). Furthermore, sequencing of the complete 16S-23S internal-transcribed spacer (ITS) was performed for 10 strains by using the primers 1406F (31) and 23Sr (16). A set of reference ITS sequences from 20 other PnecC strains and 19 other non-PnecC *Polynucleobacter* strains was established for the proper alignment and analysis of the obtained sequences. Align-

TABLE 2. Primers developed for the screening of the clone library

Primer	Type	Sequence (5'-3')	Length (nt)	Melting temp (°C)	Target	Specificity
19F <sup>a</sup>	Forward	MTG GCT CAG ATT GAA CGC T	19	55.6	16S rRNA gene	Many bacteria
PnecCr-4 <sup>b</sup>	Reverse	AAC GAG CAC CAT TGC TAG T	19	54.5	16S-23S ITS	PnecC
PnecCr-5 <sup>b</sup>	Reverse	AAC GAG CAC CAT TGC TAG Y	19	55.6	16S-23S ITS	PnecC
PnecCG1F	Forward	TAA ATG TCA AAA CTA AGC GAT CTA A	25	54.8	16S-23S ITS	ITS genotype 1 (PnecC)
PnecCG1+2strF	Forward	TAG AGA AAA GAT GCT GAA TCC TA	23	55.3	16S-23S ITS	ITS genotypes 1 and 2 and a few other genotypes (PnecC)
PnecCG2F	Forward	AGA CCC ACC AAT CAG CGT	18	56.0	16S-23S ITS	ITS genotype 2 (PnecC)
PnecCG3F	Forward	ACC CAC CAT CAG CAG CA	17	55.2	16S-23S ITS	ITS genotype 3 (PnecC)

<sup>a</sup> Primer targets a conserved sequence close to the 5' end of the 16S rRNA gene. Melting temperature was optimized for combination with primers PnecCr-4 and PnecCr-5.

<sup>b</sup> Primers PnecCr-4 and PnecCr-5 differ in only 1 nucleotide and target the same position of the ITS. Primer PnecCr-4 may not work in the case of one PnecC genotype.

ment of the ribosomal sequences was performed by using the ARB software package (32). Phylogenetic trees were constructed as described previously (23).

**Preliminary taxonomic classification of the isolated strains.** The isolated strains are closely related to the endosymbiotic type strain of *Polynucleobacter necessarius* (16S rRNA similarity, 99.2 to 99.4%). This species was described as an obligate endosymbiont of a ciliate (26). Despite the close phylogenetic relationship, the isolates were preliminarily not classified as *Polynucleobacter necessarius* strains due to the potential differences in lifestyle (obligate endosymbionts versus potentially completely free-living planktonic). Instead, the isolates are considered members of the subcluster C of the *Polynucleobacter* cluster (PnecC) (23). For the sister group of PnecC, the subcluster D of the *Polynucleobacter* cluster, the term PnecD is used.

**Construction and analysis of a clone library.** For construction of a clone library of fragments of the ribosomal operons of bacteria, water samples (200 ml) were taken on 15 October 2003, filtered on a membrane filter (pore size, 0.2 µm; diameter, 47 mm; Millipore), and stored at -70°C until processing. For the extraction of DNA, the FastDNA kit and the FastPrep instrument (Q-Biogene) were used. Fragments of the ribosomal operons were amplified with primers 27F (31) and 23Sr (16). Both primers are universal bacterial primers, which bind to positions 8 to 27 (*Escherichia coli* numbering) of the 16S rRNA gene and positions 130 to 114 (*E. coli* numbering) of the 23S rRNA gene, respectively. Amplification was performed in a Primus 96<sup>plus</sup> thermocycler (MWB-Biotech) in 50-µl reaction mixtures containing PCR buffer, approximately 100 ng of DNA, 200 µM concentrations of deoxynucleoside triphosphates (dNTPs), 2 mM MgCl<sub>2</sub>, 1.25 U of *Taq* DNA polymerase (QIAGEN), and 0.2 µM concentrations of (each) primer. The cycling conditions were initially denatured at 94°C for 3 min, which was followed by 30 cycles at 94°C for 1 min, 53.0°C for 1 min, and 72°C for 2 min. Cycling was finished with an extension step of 10 min at 72°C. The PCR products were purified with the QIAquick PCR purification kit (QIAGEN), ligated in pDrive cloning vector (PCR Cloning plus kit; QIAGEN, Hilden, Germany), and cloned into competent *E. coli* cells according to the instructions by the manufacturer. The harvested clones were screened for the length of the inserts, and only clones with inserts of >1,500 bp were considered for further analysis. For the detection of PnecC clones, the library was screened by using primer pair 19F/PnecCr-4 (annealing temperature, 63.0°C) (Table 2). The reverse primer PnecCr-4 is specific for ITS sequences of PnecC bacteria. For identifying different ITS genotypes, the PnecC clones were further screened by using the restriction enzyme EcoNI. Only 3 out of the 21 ITS genotypes (strains with different ITS sequences) known from PnecC bacteria possess one restriction site (instead of two) in the cloned 27F/23Sr fragment. Two out of these three ITS genotypes are exclusively known from the investigated pond (isolated strains). Further differentiation of PnecC ITS genotypes was performed by using ITS genotype-specific primer pairs (Table 2). The two primer pairs PnecCG1F/23Sr (annealing temperature, 62.5°C) and PnecCG1+2strF/PnecCr-5 (annealing temperature, 66.0°C) were used for the detection of clones belonging to ITS genotypes 1. The first primer pair is specific for the ITS genotype 1, while the second primer pair targets ITS genotype 1 and two more genotypes (out of 21) affiliated with PnecC. The two forward primers target sequences located at different positions of the ITS sequences. Primers PnecCG2F and PnecCr-5 (annealing temperature, 67.0°C) were used for the detection of ITS genotype 2 clones, and primers PnecCG3F and PnecCr-5 (annealing temperature, 65.0°C) were used for the identification of ITS genotype 3 clones. Theoretical evaluation of the specificity of the newly designed primers (by BLAST) revealed for all primers no 100% match to any known sequence. PCR conditions for the newly designed

primers were optimized by using a gradient temperature cycler (Eppendorf) and template DNA from target and nontarget organisms. Besides the annealing temperatures, PCR conditions were as described above.

Twenty-five clones, including representatives of all the PnecC genotypes detected in the clone library, were sequenced. The sequences were aligned and analyzed as described for the sequences of the isolates.

**Whole genome fingerprinting of cultured strains.** The three different techniques enterobacterial repetitive intergenic consensus (ERIC) PCR, repetitive extragenic palindromic-PCR (REP-PCR), and random amplification of polymorphic DNA (RAPD) were used for fingerprinting of the isolates obtained from the investigated pond. (i) For ERIC, the primers ERIC1R and ERIC2 (12) were used. The 20-µl PCR mixture contained 0.5 U *Taq*, 2.5 mM MgCl<sub>2</sub>, 200 µM concentrations of dNTPs, 2 µl 10× PCR buffer, 0.2 µM concentrations of (each) primer, and 1 µl template DNA. The cycling conditions were 3 min at 94°C; 41 cycles with 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; and a final cycle at 72°C for 10 min. (ii) For REP-PCR, the primers REP1R and REP2-I (12) were used. The 20-µl PCR mixture contained 2 U *Taq*, 2.5 mM MgCl<sub>2</sub>, 200 µM concentrations of dNTPs, 2 µl 10× PCR buffer, 0.25 µM concentrations of (each) primer, and 1 µl template DNA. Cycling conditions were 6 min at 94°C; 41 cycles with 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C; and a final cycle at 72°C for 10 min. (iii) Three different RAPD fingerprintings were performed by using the primers A, B, and C (49). The PCR conditions were according to the protocol by Ziemke et al. (49).

**Nucleotide sequence accession numbers.** Twenty-five 16S rRNA gene and ITS1 sequences of clones and isolates obtained from the investigated pond were deposited under accession numbers AJ879778 to AJ879802. The submission of 10 ITS1 sequences, as well as several partial 16S rRNA sequences identical with one or more other sequences, was omitted.

## RESULTS

**Basic chemophysical characteristics of the investigated pond.** The water of the pond was characterized during the investigation period by a low conductivity (7 to 52 µS cm<sup>-1</sup>) and an acidic pH (4.3 to 6.0). Measured water temperatures ranged from 0.1 to 17.3°C, and the oxygen saturation was usually around 100% but dropped during the ice-covered period (Fig. 1) to <1% saturation (February 2004).

**Determination of PnecC numbers by FISH.** Water samples taken in the period from September 2003 to October 2004 were investigated by FISH. Probe PnecC-16S-445, which is specific for the narrow, species-like *Polynucleobacter* subcluster C (PnecC, 98.5% minimum 16S rRNA gene sequence similarities), detected cell numbers in the range of 0.02 × 10<sup>6</sup> to 1.12 × 10<sup>6</sup> cells ml<sup>-1</sup> (Fig. 1). These numbers equal the relative abundances of 4 to 59% of the total numbers of bacterioplankton cells. All of the detected cells were freely suspended planktonic bacteria, and no probe-positive cells were found to be located inside ciliate cells. Probe PnecC-23S-166, which is also

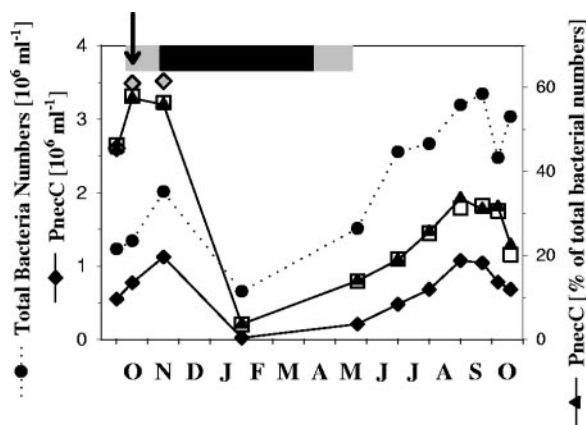


FIG. 1. Annual cycle of PnecC population dynamics in the water column of the investigated dystrophic pond in the period from September 2003 to October 2004. Relative abundances of the PnecC population were obtained by using probes PnecC-16S-445 (16S rRNA targeted [black triangles]) and PnecC-23S-166 (23S rRNA targeted [gray diamond], only data for 2003). The entire *Polynucleobacter* cluster plus a few related sequences outside the cluster were targeted with probe BET2-870 (open squares). The absolute numbers of the PnecC population are based on the data obtained with probe PnecC-16S-445. The period of ice cover on the pond is depicted by the gray (temporary, e.g., overnight) and black (presumably permanent-cover) parts of the horizontal bar. The arrow indicates the date of sampling for construction of the clone library and the establishment of the culture collection.

PnecC specific but targeting the 23S rRNA, was applied to samples from 2003 and detected very similar percentages of total bacterial numbers. Probe PnecABD-445, specific for the other three subclusters of the *Polynucleobacter* cluster, detected no bacteria in any of the samples. The two probes PnecD1-181 and PnecD2-181, which together cover almost the entire subcluster D, were applied to the sample from 15 October 2003 and also detected no bacteria. Application of the relatively broad probe BET2-870, targeting the entire *Polynucleobacter* cluster and a few related sequences outside

the cluster, resulted in very similar data as obtained by probes PnecC-16S-445 and PnecC-23S-166 (Fig. 1). Obviously, only *Polynucleobacter* bacteria belonging to PnecC could be detected in the pond during the 13-month investigation period. Results from FISH with probes targeting large phylogenetic groups support the image of the large contribution of PnecC bacteria to total bacterioplankton (Fig. 2).

**Isolation and genotyping of PnecC strains.** In total, 22 strains belonging to PnecC were isolated from the water sample taken on 15 October 2003. The 16S rRNA genes of all strains were partially or almost completely sequenced. Only three genotypes could be found among the isolated strains. The almost complete sequences differed only in four sequence positions (minimum sequence similarity, 99.80%). Sequencing of the 16S-23S ITS sequences of representatives of each of the three genotypes did not further increase the revealed genetic diversity of the isolates (Fig. 3). All investigated strains of the same 16S rRNA genotype possessed identical ITS sequences (including two tRNA genes) of 515 or 519 nucleotides in length. The ITS sequences of the three genotypes differed in 33 positions (including insertions and deletions). Both the three 16S rRNA gene sequences and the three ITS sequences are novel genotypes different from the genotypes previously found among investigated PnecC isolates (23; unpublished data).

By fingerprinting the 22 strains with ERIC, REP-PCR, or RAPD (three different RAPD primers were used), only three genomic genotypes could be detected. All applied fingerprinting methods could not reveal any genotypic differences between strains of the same ITS genotype but always revealed pronounced differences between strains of different ITS genotypes (Fig. 4). Thus, the three genomic genotypes revealed by fingerprinting were in perfect congruence with the ribosomal genotypes.

**Analysis of the clone library.** A clone library containing 103 clones of ribosomal operon fragments obtained from the water sample taken on 15 October 2003 was established. The library was systematically screened for PnecC sequences, as well as for

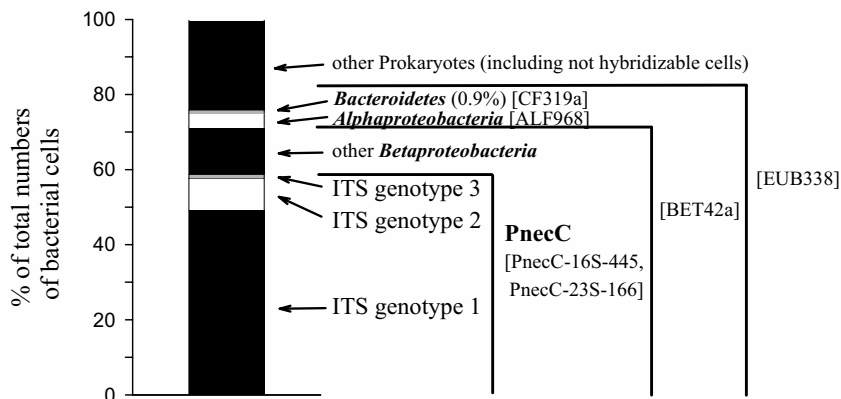


FIG. 2. Composition of the bacterioplankton community in the investigated dystrophic pond on 15 October 2003. The presented percentages are based mainly on results from hybridization with FISH probes. The data for the *Polynucleobacter* subcluster C population are the average data from hybridization with two different probes (probe BET2-870 also resulted in the detection of ~60% of the DAPI-stained cells). The percentage of other *Betaproteobacteria* is the difference between counts with probe BET42a and the percentage of *Polynucleobacter* bacteria. The fraction of other prokaryotes includes bacteria not hybridized with the used group-specific probes and the 19% of DAPI-stained cells which gave no detectable signals with probe EUB338. Percentages of *Polynucleobacter* ITS genotypes represent estimates based on the analysis of the clone library. *Gammaproteobacteria* were detected but could not be enumerated due to very low cell numbers. Names of probes are shown in square brackets.

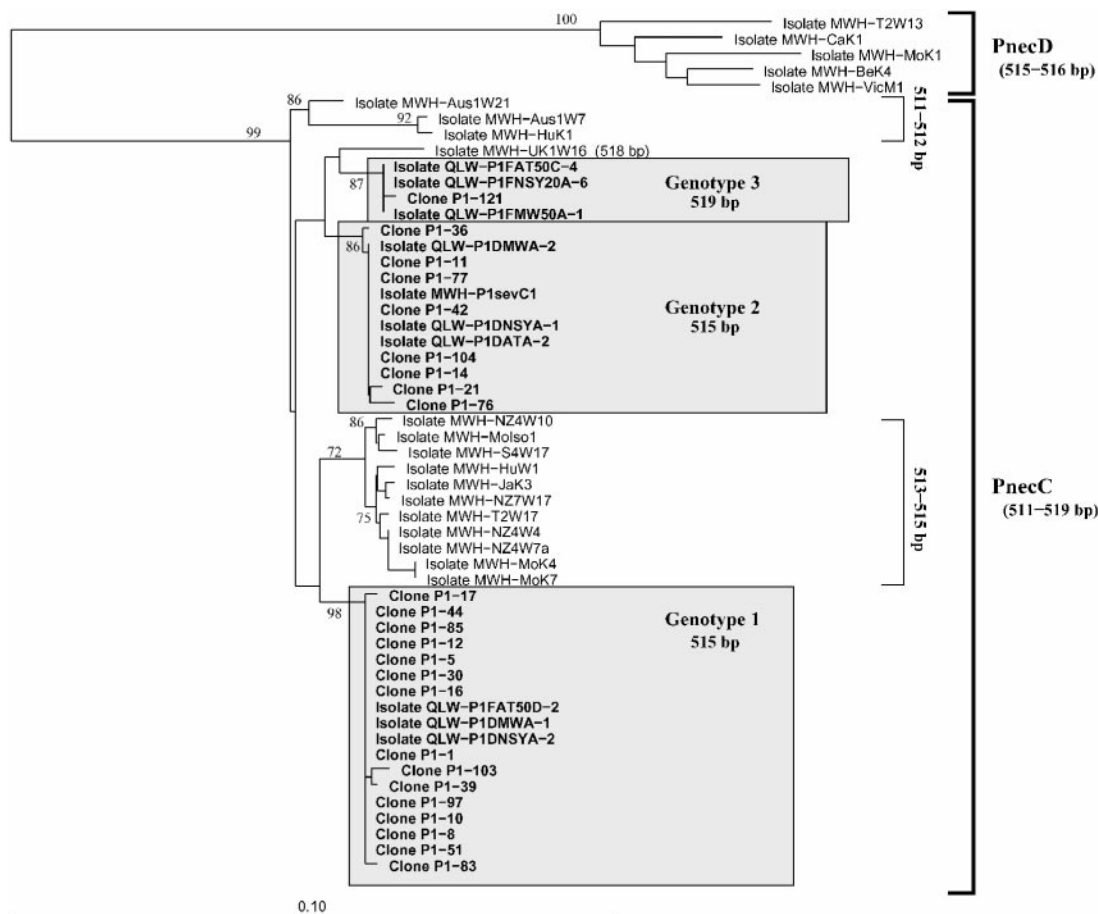


FIG. 3. Neighbor-joining tree showing the phylogenetic relationships of isolated and uncultured strains affiliated with PnecC and PnecD. The tree was calculated with complete 16S-23S ITS sequences (511 to 519 nucleotides). The length of the complete ITS sequences (including two tRNA genes) is indicated for single sequences or for groups of sequences. The two subclusters, PnecC and PnecD, are indicated by the outermost right brackets. Isolates and clones of the three genotypes found in the investigated pond are indicated by gray boxes. The three ITS genotypes known from the investigated pond and the other 13 ITS genotypes shown together represent 76% of the currently known ITS genotypes affiliated with PnecC. The ITS sequence (510 bp) of *Ralstonia solanacearum* (NC\_003295) served as the outgroup (data not shown). Bootstrap (1,000 iterations) values of >70% are shown. The scale bar indicates 10% estimated sequence divergence. Clone P1-31 (genotype 1) is not shown in the tree. Its ITS sequence is identical with the sequences of the genotype 1 isolates, as well as with the majority of the genotype 1 clones.

different PnecC genotypes. In total, 56 PnecC clones were detected. Thus, 54.4% of the total number of clones was comprised of PnecC clones, which is close to the 58.8% (standard deviation, 1.9; results from hybridizations with three different FISH probes) contribution of PnecC cells to the total number of bacterioplankton cells.

Screening of the clone library for different PnecC ITS genotypes by restriction fragment length polymorphism analysis and by four PCR probes specific for the three ITS genotypes known from the isolates resulted in the detection of only three ITS genotypes. Based on this approach, each PnecC clone could be assigned to one of the three ITS genotypes known from the isolates obtained from the same habitat at the same time. No contradiction between the results obtained by the two screening methods was observed. For further confirmation, 25 PnecC clones (including all clones assigned by screening to genotypes 2 and 3, as well as 16 clones assigned to genotype 1) were selected for complete ITS sequencing, as well as partial or complete 16S rRNA gene sequencing. Sixty-seven percent of

the analyzed cloned ITS sequences was identical with the sequences of isolates obtained from the same water sample. The other sequences differed in one to three sequence positions (0.2 to 0.6% of sequence positions). In phylogenetic analysis, these slightly different sequences clearly clustered with the three ITS genotypes of the isolates (Fig. 3). None of the observed sequence differences within a group of ITS genotypes was found in more than one sequence. In order to identify potential PCR artifacts among the sequence differences between cloned sequences and directly sequenced isolates of the same ITS genotype, the cloned ITS sequences were compared with a large set of PnecC and PnecD reference sequences. These reference sequences were obtained by direct sequencing from cultured representatives of the two *Polynucleobacter* subclusters (Table 3). Only 1 of the 13 identified potential PCR artifacts was located at a sequence position, which is variable within PnecC. The other 12 potential artifacts were located in positions which were found to be invariable within PnecC or even within both subclusters. Based on this sequence compar-

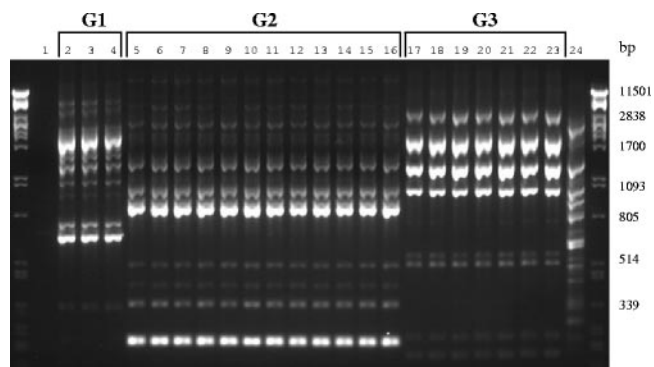


FIG. 4. Genomic fingerprints of PnecC strains isolated from the dystrophic pond. The presented RAPD fingerprints were obtained by using primer A (49). Use of two other RAPD primers, as well as the application of two other fingerprinting methods (ERIC and REP-PCR), did not result in differences in the fingerprints of strains of the same ITS genotype. The isolates were assigned to the respective genotypes based on their ribosomal sequences. Relatively large amounts of PCR products were applied to the gel to make weak bands also visible. Lane 1, negative control (no template DNA); lanes 2 through 4, genotype 1 (G1) strains; lanes 5 through 16, genotype 2 (G2) strains; lanes 17 through 23, genotype 3 (G3) strains; lane 24, *E. coli*; outermost left and right lanes, lambda ladder.

ison, we assume that the 25 cloned ITS sequences analyzed represent only three ITS genotypes.

**Reconstruction of the PnecC population structure and the bacterioplankton community structure.** The cloned ribosomal fragments of the three PnecC ITS genotypes differ in length by only 3 nucleotides (1 bp in 16S rRNA genes and 4 bp in ITS sequences); therefore, we do not expect significant differences in PCR amplification of these sequences. If one further assumes for these very closely related strains no pronounced differences in ribosomal copy numbers per cell, as well as no genotype-specific differences in the efficiency of DNA extraction, then the established clone library could be used for the estimation of the PnecC population structure in the investigated pond. Quantitative analysis of the screening of the PnecC clones revealed that 84% of the PnecC clones belong to ITS genotype 1, 14% to ITS genotype 2, and only 2% to ITS

genotype 3. This indicates a strongly uneven genotypic structure of the PnecC population at the day of sampling.

For estimation of the contribution of the single PnecC ITS genotypes to the total bacterioplankton, their potential under representation in the clone library was estimated by comparison of the PnecC percentage determined by FISH (58.8%) and the PnecC percentage in the clone library (54.4%). This approach resulted in the estimation that PnecC genotype 1 contributed ca. 50% to the total number of bacterial cells in the investigated dystrophic pond (Fig. 2).

Comparison of cloned PnecC sequences with sequences obtained from the isolates (Fig. 3) demonstrates complete coverage of the PnecC population by cultivation. Therefore, the genomic fingerprinting of the isolates provides insights into the population structure beyond the phylogenetic resolution of ribosomal markers. The detection of only three genotypes by fingerprinting indicates a low microdiversity of the PnecC population even on the genome level.

## DISCUSSION

In order to verify the surprisingly large contribution of the PnecC population to the bacterioplankton in the investigated habitat, a set of nested FISH probes was applied. Two independent probes specific for subcluster PnecC, as well as a broader probe (7) specific for the entire *Polynucleobacter* cluster (23) and a few sequences outside of this cluster, provided very similar results. Application of a probe covering the entire non-PnecC part of the *Polynucleobacter* cluster resulted in no detections. These consistent results clearly verify the dominance of the PnecC population; however, we cannot exclude an underestimation of the PnecC numbers by the presence of PnecC cells giving only undetectable hybridization signals.

The difference of 4% in the contribution of PnecC to total bacterial numbers (FISH) and the total number of clones in the library could be a result of several factors (14, 41, 46). On the other hand, we do not expect a strongly biased representation of the different PnecC ITS genotypes in the clone library because we assume neither significant differences in DNA extraction efficiency for the closely related PnecC strains nor strong differences in rRNA gene copy numbers, or a strong

TABLE 3. Analysis of the 25 ITS sequences affiliated with PnecC obtained from the established clone library for potential PCR artifacts<sup>a</sup>

Analyzed ITS sequences (source)	Affiliation of sequences	No. of sequences	No. of analyzed sequence positions	Potential PCR artifacts (no. of sequence positions)	No. of potential artifacts in positions:		
					Conserved in PnecC and PnecD	Conserved in PnecC	Variable within PnecC and PnecD
PnecC reference sequences <sup>b</sup>	PnecC	34	17,511		No variation <sup>d</sup>	No variation <sup>e</sup>	Variable <sup>f</sup>
PnecD reference sequences <sup>c</sup>	PnecD	18	9,279		No variation <sup>d</sup>	No variation <sup>e</sup>	Variable <sup>f</sup>
Clones (this study)	PnecC	25	12,879	13	11	1	1

<sup>a</sup> Sequence positions, which differed in the cloned sequences from sequences of isolates belonging to the same ITS genotype, were considered potential PCR artifacts. In total 13 positions, i.e., 0.1% of the total number of cloned ITS sequence positions with potential PCR artifacts, were identified. These positions were compared with the homologous positions in a set of 52 reference sequences affiliated with subclusters C and D of the *Polynucleobacter* cluster. All reference sequences were obtained by direct sequencing. Only one potential PCR artifact was found to be located in a variable sequence position. The other sequence differences were located in sequence positions absolutely invariable in both subclusters or at least within PnecC.

<sup>b</sup> Sequences from directly sequenced isolates and endosymbionts belonging to *Polynucleobacter* subcluster C (PnecC), including isolates obtained from the dystrophic pond.

<sup>c</sup> Sequences from directly sequenced isolates belonging to *Polynucleobacter* subcluster D (PnecD).

<sup>d</sup> No variation within PnecC and PnecD, and identical in both subclusters.

<sup>e</sup> No variation within PnecC and PnecD, but not identical in both subclusters.

<sup>f</sup> Variable within PnecC and PnecD.

difference in the template-to-product ratio in the PCR of PnecC sequences. Polz and Cavanaugh (38) compared template and product ratios in PCR amplifications of the ribosomal genes of closely related species and observed shifts by factors of 1.0 to 1.8. The sequences of the different PnecC ITS genotypes are much more similar to each other than the sequences of these closely related species; however, the frequencies of the ITS genotypes in the clone library differed by factors of 5.9 and 47.0. Thus, it appears to be unlikely that the uneven ITS genotype distribution in the clone library is predominantly a result of PCR bias. We assume that the ITS genotype frequencies observed in the established clone library provide a semiquantitative image of the genotypic structure of the investigated PnecC population.

**Large PnecC numbers in the pelagic zone of the investigated pond.** To our knowledge, the absolute numerical dominance of a species-like bacterial population in a nonextreme aquatic habitat, as well as the occurrence of such a planktonic population with cell numbers of  $>1 \times 10^6 \text{ ml}^{-1}$ , was not reported previously. Bacterial groups demonstrated to occur in nonextreme habitats with high relative numbers represented much broader phylogenetic groups (7, 36). For instance, the well-investigated SAR11 cluster, which comprises up to 50% and, on average, 30% of bacterioplankton in marine surface waters (36), represents a broad phylogenetic group with a minimum 16S rRNA sequence similarity of 88.8% (PnecC, 98.5%). This low similarity value indicates that this cluster potentially harbors several species belonging to one or several genera.

The PnecC group is a phylogenetically narrow, but potentially ecologically diverse, group (23, 26, 44). Strains affiliated with this group are thought to be obligate endosymbionts of at least two benthic ciliate species living in fresh and brackish water (26, 44). We demonstrated by FISH the presence of free-living PnecC cells in the pelagic zone of a freshwater habitat. This indicates that many or all of the environmental PnecC sequences collected from the pelagic zones of freshwater habitats (7, 10, 11, 21, 27, 50) originate from free-living cells; however, it is still not known if these cells represent completely free-living strains or facultative endosymbionts of ciliates. The numerically significant contribution of free-living stages of facultative endosymbionts or facultative parasites of protists (e.g., *Legionella* spp.) to total numbers of bacterial communities has not been observed (17). However, detailed studies on PnecC bacteria have to reveal whether the endosymbiotic stages (26, 44) and the observed free-living stages represent two completely independent lifestyles or only two stages of the same lifestyle (i.e., facultative endosymbionts). This knowledge will be essential for the understanding of the reasons for the observed numerical dominance of the PnecC bacteria.

**Low microdiversity of the PnecC population.** Only a few studies have revealed the microdiversity of free-living bacterial populations (1, 29, 42, 45). The investigated PnecC population differed from these populations in a low microdiversity and a strongly uneven population structure. The observed low microdiversity may reflect a low overall genotype richness of the PnecC population in the pond or may be a snapshot of a more diverse population with a dynamic structure. Low genotype richness of the PnecC population would be in conflict with the "everything is everywhere" hypothesis (4, 15), which assumes

that free-living microorganisms are easily dispersed. Therefore, the presence of only a small fraction of the existing PnecC ITS genotypes would not be expected. On the other hand, dispersal restrictions exist for other aquatic bacteria, which result in the appearance of biogeography in these species (20, 22); therefore, we cannot exclude the existence of dispersal restrictions in PnecC bacteria. Further on, the frequencies of potential invasion and the extinction of genotypes could be of importance in small habitats occupied by relatively small populations. The PnecC population in the pond may get bottlenecked during ice-covered periods with oxygen depletion. Bottlenecking in combination with low invasion rates may result in limited genotype richness. Obviously, insights into the dynamics of population structures, as well as a detailed knowledge of the dispersal of free-living aquatic bacteria, are necessary for a better understanding of the intraspecific diversity of bacterial populations in freshwater.

**Uneven structure of the PnecC population.** Thompson et al. (42) estimated that a marine *V. splendidus* population consisted of >1,000 unique genotypes and deduced from allele frequencies within the investigated sample of 232 cultured strains a relatively even population structure. The strongly uneven population structure observed in the PnecC population could either be the result of genetic drift or be the result of ecological selection of particular genotypes. Explanation by genetic drift would require a small effective population size, which is usually not given in populations of free-living bacteria but was demonstrated in populations of endosymbiotic bacteria (47). As we do not know the intensity of gene flow (i.e., dispersal of genotypes) between the PnecC population of the investigated pond and the populations of other habitats, it is not possible to rule out the influence of genetic drift. On the other hand, the observed uneven population structure could be explained by genotype-specific selection in predation or genotype-specific differences in the ability to utilize available substrates. Pronounced differences in grazing mortality between four closely related ( $\geq 99.6\%$  16S rRNA similarity) strains affiliated with the *Polynucleobacter* subcluster D (PnecD) were observed (5). Strain-specific grazing mortality differed under predation by the same flagellate strain by up to threefold, and the grazing mortality of the same bacterial strain differed under grazing by three closely related flagellates (100% identity of the 18S rRNA genes) by up to fourfold. Intraspecific differences in grazing sensitivity were also observed for *Hylemonella gracilis* strains (48).

**Ecological importance of microdiversity.** Thompson et al. (42) suggested that much of the huge genotypic diversity observed in a *V. splendidus* population is ecologically neutral, i.e., redundant. The observed large contribution of a PnecC population with a low microdiversity demonstrates that ecologically successful bacterial populations do not need to possess a high genetic diversity. A high intraspecific diversity could result in a high degree of ecological differentiation within the population, which could contribute to the ecological success of the population. The observed combination of a low intraspecific diversity and high contribution to the total bacterioplankton community may support the assumed ecological redundancy within genetically diverse populations (42). However, the observed coexistence of only a few of the known PnecC genotypes leaves the possibility open that some PnecC genotypes differ in

their ecological adaptation and occupy partially separated ecological niches. Such ecological differences in closely related bacteria are known from other bacterial groups (6, 24, 28).

For a deeper understanding of the ecology and function of aquatic bacteria, the ecological meaning of intraspecific diversity (microdiversity) and the potential intraspecific ecological diversification in bacterial populations and species have to be fully revealed.

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