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Development and Application of Monoclonal Antibodies for In Situ Detection of Indigenous Bacterial Strains in Aquatic Ecosystems

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Strain-specific monoclonal antibodies (MAbs) were developed for three different bacterial isolates obtained from a freshwater environment (Lake Plußsee) in the spring of 1990. The three isolates, which were identified by molecular methods, were as follows: *Cytophaga johnsonae* PX62, *Comamonas acidovorans* PX54, and *Aeromonas hydrophila* PU7718. These strains represented three species that were detected in high abundance during a set of mesocosm experiments in Lake Plußsee by the direct analysis of low-molecular-weight RNAs from bacterioplankton. We developed one MAb each for the bacterial isolates PX54 and PU7718 that did not show any cross-reactivity with other bacterial strains by immunofluorescence microscopy. Each MAb recognized the general lipopolysaccharide fraction of the homologous strain. These MAbs were tested successfully for their ability to be used for the in situ detection and counting of bacteria in lake water by immunofluorescence microscopy. During the spring of 1993, *A. hydrophila* PU7718 showed a depth distribution in Lake Plußsee with a pronounced maximum abundance at 6 m, whereas *Comamonas acidovorans* PX54 showed a depth distribution with a maximum abundance at the surface. The application of these MAbs to the freshwater samples enabled us to determine the cell morphologies and microhabitats of these strains within their natural environment. The presence of as many as 8,000 cells of these strains per ml in their original habitats 3 years after their initial isolation demonstrated the persistence of individual strains of heterotrophic bacteria over long time spans in pelagic habitats.

Antibodies as used in immunological test systems are powerful tools for the specific detection and identification of bacteria. There is a wealth of information on the successful applications of antibodies in the medical and diagnostic fields (17, 21, 27). In contrast, only limited information is available on the use of antibodies for the identification of bacteria in the area of microbial ecology, i.e., for the detection of bacteria in samples collected from natural environments. One reason for the limited application of antibodies in past years has been the rapid development of molecular probes based on complementary DNA sequences targeted against rRNA and their increased utilization in the detection and taxonomic classification of bacteria in the environment (1, 11, 22, 33). Nevertheless, there are several advantages in using antibodies to detect bacterial microorganisms in environmental samples. For instance, they allow the visualization of bacteria at the single-cell level under nondestructive conditions and independently of the growth rate of the cell (8, 25). This type of visualization leads to the possibility of estimating the number of bacteria in distinct compartments of a given habitat and, because of the preservation of the in situ structure of natural samples, permits better insight into the microenvironment in which bacteria live (4, 7).

One of the most important considerations in using immunological techniques for the identification of microorganisms is the possibility of the antibodies cross-reacting with bacterial antigens other than the target, e.g., conserved carbohydrogenic or protein epitopes on the surfaces of microorganisms. To circumvent this problem, it is possible to select highly specific monoclonal antibodies (MAbs) against isolate-specific epitopes from an immunized mouse by a well-described hybridoma technique (18). This approach is more laborious than that using polyclonal antibodies but has the advantage of better reproducibility of results after its application to environmental samples, because the antibody is well-defined and can be produced reliably in unlimited amounts. Furthermore, the specificities of the MAbs must be assessed with a wide taxonomic range of reference bacteria comprising closely related strains, as well as distantly related strains.

This work deals with the development and application of MAbs against three different bacteria isolated from a freshwater environment (Lake Plußsee in northern Germany) and their use for the in situ detection of these individual strains in samples collected from the source of their original isolation. A fast and efficient screening strategy leading to strain-specific MAbs for environmental isolates was developed. The bacterial strains used as antigens for the development of the MAbs were isolated during mesocosm experiments (3) and were observed in increased abundance within the bacterioplankton community under the conditions established in the mesocosms (15). Two of the strains (PX62 and PX54) came from samples collected directly from water of the mesocosms, whereas the third strain was isolated from lake water outside of the mesocosms. As determined by molecular identification methods, the three isolates represented very different taxonomic groups, namely, (i) the phylum containing flavobacteria, bacteroides, and cytophaga for *Cytophaga johnsonae* PX62; (ii) the β-subclass of the *Proteobacteria* for *Comamonas acidovorans* PX54; and (iii) the γ-subclass of the *Proteobacteria* for *Aeromonas hydrophila* PU7718. In addition to the three isolates used for the generation of the MAbs, a large set of isolates from Lake Plußsee and the mesocosms was obtained and screened for closely related genotypes by low-molecular-weight (LMW)-RNA pro-

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TABLE 1. List of bacterial strains used in this study from Lake Plußsee[a]

Strain	Lake Plußsee mesocosm or depth (m)	Isolation date[b]
Comamonas acidovorans PX54	Mesocosm 8	28 May 1990 (13)
Cytophaga johnsonae (type II) PX62	Mesocosm 8	31 May 1990 (16)
Cytophaga johnsonae (type I) PX3	Mesocosm 7	18 May 1990 (3)
Cytophaga johnsonae (type I) PX20	Mesocosm 7	28 May 1990 (13)
Cytophaga johnsonae (type II) PX60	Mesocosm 8	31 May 1990 (16)
Cytophaga johnsonae (type II) PX41	Mesocosm 8	25 May 1990 (10)
Cytophaga johnsonae (type II) PX58	Mesocosm 8	31 May 1990 (16)
Cytophaga johnsonae (type II) PX51	Mesocosm 8	28 May 1990 (13)
Cytophaga johnsonae (type II) PX23	Mesocosm 7	31 May 1990 (16)
Cytophaga johnsonae (type II) PX43	Mesocosm 8	25 May 1990 (10)
Cytophaga johnsonae (type II) PX25	Mesocosm 7	31 May 1990 (16)
Cytophaga johnsonae (type II) PX28	Mesocosm 7	31 May 1990 (16)
Cytophaga johnsonae (type II) PX46	Mesocosm 8	25 May 1990 (10)
Cytophaga johnsonae (type II) PX65	Mesocosm 8	28 May 1990 (13)
Cytophaga johnsonae (type II) PX62	Mesocosm 8	31 May 1990 (16)
Aeromonas hydrophila PU7718	1	12 March 1990
Aeromonas hydrophila PU77-8	1	12 March 1990
Aeromonas hydrophila PU69-8	1	15 January 1990
Aeromonas hydrophila PU69-7	1	15 January 1990
Aeromonas hydrophila PU84-20	1	18 April 1990
Aeromonas hydrophila PU84-24	1	18 April 1990
Isolate PU66-15 (related to PX54)	1	11 December 1989

[a] Strains from Lake Plußee were isolated during mesocosm experiments and from lake water samples. Mesocosm 7 was an unamended control: mesocosm 8 was amended with organic nutrients (final concentration, 2.8 mg of carbon per liter). For details of the mesocosm experiments, see references 3, 4, and 15. We also used reference strains from the Deutsche Stammsammlung für Mikroorganismen as follows: *A. hydrophila* subpp. *hydrophila* DSM 30187^T, *A. hydrophila* subsp. *androgena* DSM 30188^T, *A. hydrophila* subsp. *proteolytica* DSM 30189^T, *Aeromonas punctata* DSM 30190, *Aeromonas media* DSM 4881^T, *Aeromonas schubertii* DSM 4882^T, *C. johnsonae* DSM 2064^T, and *C. johnsonae* DSM 425.

[b] The number of days after the start of the mesocosm experiment is given in parentheses.

filing (14, 16). The analysis of a combination of reference strains and closely related isolates from the same environment enabled us to establish the specificities of the generated MAbs and to assess the abundance and distribution of the isolates in their habitat.

MATERIALS AND METHODS

Isolation of strains and genotypic identification. All lake water samples and strains were obtained from Lake Plußsee, one of the best-studied freshwater environments (23). The bacterial strains, designated PX54 and PX62, were isolated on CPS (Bacto Casein, peptone, starch, glycerol, minerals, 1.5% agar [6]) agar plates in May 1990 during experiments in lake water mesocosms as described previously (15). Strain PU7718 was isolated on 12 March 1990 directly from lake water at a 1-m depth on CPS medium. All water samples were taken in sterile glass bottles with a Sorokin sampler. These bacterial strains belonged to the species *A. hydrophila* (PU7718), *C. johnsonae* (PX62), and *Comamonas acidovorans* (PX54). *Comamonas acidovorans* PX54 was identified by comparative 16S rRNA gene sequence analysis (99.8% sequence similarity to the nearly complete sequence of the type strain [about 1,450 bp] [22a]). Identification of the other two isolates was done by LMW-RNA profiling (14). LMW-RNA profiles of the two identified isolates, determined with long-range gels used according to the method of Höfle and Brettar (16), showed no differences from the profiles of the type strains of the relevant species. Approximately 700 additional isolates from Lake Plußsee and the mesocosm experiments were obtained in 1989 and 1990 by the described isolation procedure. All isolates were screened genotypically by LMW-RNA profiling to detect related genotypes of the three strains used for the generation of MAbs. The characteristics of the resulting isolates, i.e., the same species as the three strains used for immunization, as well as those of reference strains are summarized in Table 1 (the strain designations beginning with PU and PX indicate isolates from Lake Plußsee and the mesocosm experiments, respectively).

Culture conditions. The isolates were freshly cultured in liquid medium (8 g of nutrient broth per liter; Difco Corp.) overnight at room temperature and used either for immunizations or for screening of hybridoma supernatants. The bacterial strains used for testing cross-reactivity (listed in Table 1) were cultivated on agar plates containing 8 g of nutrient broth (Difco Corp.) or CPS medium per liter and 1.5% agar (Difco Corp.). All strains were stored as stock cultures frozen in liquid medium containing 15% glycerol at −70°C.

Generation of MAbs. Bacterial cells grown overnight were harvested by centrifugation and washed three times in phosphate-buffered saline (PBS) (12). Before immunization of mice, bacterial cells were fixed with formaldehyde (final concentration, 1%) for approximately 30 min after the last washing in PBS. Approximately 10^7 cells in 10 μl mixed with 10 μl of incomplete Freund's adjuvant were subcutaneously injected in 20-μl aliquots into female BALB/c mice to start immunizations. The injections were repeated after 20 and 30 days. Finally, 4 days prior to fusion, the mice received 10^7 bacterial cells in PBS subcutaneously without adjuvant. Lymphocytes were harvested from popliteal lymph nodes and fused with the mouse myeloma cell line X63-Ag 8.536 with polyethylene glycol, as described by Köhler and Milstein (18). Hybridomas were selected in hypoxanthine-azaserine medium. Selection of positive clones was assayed by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence microscopy. Clones producing MAbs with the selected specificities were cloned by limiting-dilution technique (12, 26).

ELISA techniques. We used the bacterial strains listed in Table 1 as coating antigens for testing by ELISA. Microtiter plates (Maxisorb; Nunc Corp.) were coated with whole, live, or formalin-fixed bacteria at room temperature for 2 h. Before plates were coated, the bacteria were harvested from liquid culture, washed three times, and suspended in coating buffer at a concentration of approximately 2×10^8 cells per well. After being coated, the microtiter plates were washed with PBS supplemented with 0.05% Tween 20 (PBST). The first incubation step consisted of the addition of 50 μl of hybridoma culture supernatant and incubation for 2 h at room temperature. Unbound antibodies were removed by washing the microtiter plates three times with PBST, and subsequently, plates were incubated with peroxidase-conjugated goat anti-mouse antibodies at a dilution of 1:1,500 in PBST–5% fetal calf serum (FCS) for 2 h at room temperature. Unbound conjugate was removed by washing steps as described before. Color development was achieved by adding substrate solution (ortho-phenylene-diamine; Sigma Corp.). After 15 min, the color reaction was stopped and read at 490 nm by an ELISA plate reader. Positive and negative controls included mouse hyperimmune serum, mouse preimmune serum, and hybridoma culture medium. Antibody isotyping was performed by ELISA with isotype-specific anti-immunoglobulin conjugates, as described above.

Immunofluorescence microcopy of bacteria. Approximately 5×10^7 bacterial cells of reference strains and isolates (Table 1) were washed twice with PBS and incubated on slides precoated with poly-L-lysine (1 mg/ml) for 10 min. After a washing step and blocking with PBS–5% FCS, hybridoma supernatants were incubated for 1 h at room temperature. The slides were then washed three times with PBS and incubated for 1 h with fluorescein-isothiocyanate-conjugated anti-mouse immunoglobulin (Dianova Corp.) diluted 1:100 with PBS–5% FCS. After the washing steps were performed as described above, the slides were covered with the mounting medium Moviol (Hoechst Corp.) containing 1% DABCO (1,4-diazabicyclo[2.2.2.] octane; Sigma Corp.) to prevent fading.

Techniques for the detection of bacterial cells in water samples and microscopic analysis were adapted from those described by Hoff (13) and Enger et al.

TABLE 2. Reactivities of MAbs during primary screening against the isolates *Comamonas acidovorans* PX54, *A. hydrophila* PU7718, and *C. johnsonae* PX62 by ELISA and by an indirect immunofluorescence test with an epifluorescence microscope

MAb	ELISA reactivity (extinction, 490 nm)			Reactivity by immunofluorescence microscopy[a]		
	Comamonas acidovorans PX54	*C. johnsonae* PX62	*A. hydrophila* PU7718	*Comamonas acidovorans* PX54	*C. johnsonae* PX62	*A. hydrophila* PU7718
I4B1 (anti-PX54)	2.253	0.083	0.072	3+, RF[b]	No reaction	No reaction
I4C6 (anti-PX54)	2.380	0.126	0.093	3+[b]	No reaction	No reaction
I3A1(anti-PX54)	1.686	0.229	0.147	2+[b]	No reaction	No reaction
II1B2 (anti-PX62)	0.134	1.949	0.128	No reaction	2+[b]	No reaction
III4G8 (anti-PU7718)	0.078	0.078	2.053	No reaction	No reaction	3+, RF[b]
V3C3 (anti-PX62)	0.056	2.354	0.062	No reaction	2+	No reaction
Conjugate control	0.024	0.032	0.04	No reaction	No reaction	No reaction

[a] 2+, medium reaction; 3+, strong reaction; RF, ring fluorescence.
[b] One hundred percent of these cells were positive compared with total cell counts determined by DAPI staining.

(10). Water samples were fixed with formaldehyde (final concentration, 2%). For immunfluorescence staining, 5 ml of each sample was filtered on a black polycarbonate filter (25-mm diameter, 0.2-μm pore size; Nuclepore Corp.) and 2 ml of hybridoma supernatant, containing the primary MAb, was applied for 1 h to the filter. The filter was then washed three times with PBS, and the DTAF (dichlorotriazinylamino-fluorescein)-conjugated anti-mouse immunoglobulin antibody (polyclonal; Dianova Corp.) was applied for 1 h at a 1:100 dilution in PBS–5% FCS. After additional washing steps, as described above, the DNA-specific staining of the bacteria was done by incubating the cells with 4′,6-diamidino-2-phenylindole (DAPI) in PBS (final concentration, 6 μg/ml) for 5 min (24). For microscopic observations, membrane filters were covered with Moviol containing 1% DABCO.

Determination of numbers of bacteria in water samples. Specific staining, with formaldehyde-fixed lake water samples (final formation concentration, 2%), was carried out as described previously (4). After two additional washing steps with PBS, the filters were covered with mounting medium (Moviol containing 1% DABCO). All microscopic observations and photomicrographs were done with an epifluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany) at a magnification of ×1,000. DAPI-stained cells were observed and counted with Zeiss filter set 02 (G 365 exciter filter, FT 395 chromatic beam splitter filter, and LP 420 barrier filter). Immunofluorescent dye (DTAF)-stained cells were observed with Zeiss filter set 23 (dual-band filter set) to specifically visualize DTAF-labelled cells and to obtain additional signal from the red autofluorescence of chlorophyll *a*-containing cyanobacteria and eucaryotic algae. The numbers of bacteria were estimated from 250 different fields of the counting grid or from two enumeration stripes on the polycarbonate filter membrane. By this counting procedure for immunofluorescent cells and filtration of up to 30 ml of lake water, the detection limit was determined to be 20 cells/ml. The coefficient of variation was in the range of 20% for immunofluorescent cell counts >500 cells/ml and increased up to 60% for cell counts approaching the detection limit. DAPI-stained cells were counted with an average coefficient of variation of 15%.

SDS-PAGE and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (19). Twenty-five milliliters of an overnight culture was washed twice in PBS. After centrifugation, bacteria were lysed in 1 ml of SDS-PAGE sample buffer and boiled for 10 min prior to electrophoresis. After electrophoretic separation on a 12.5% acrylamide gel (100 V, 15 mA), the bands were stained with Coomassie brilliant blue. For the analysis of lipopolysaccharide (LPS), proteinase K (1 mg for 1 ml of sample) was used, for 1 h at 60°C, for the digestion of proteins. Transfer of the electrophoretically separated bands to a nitrocellulose membrane (Bio-Rad Corp.) was performed according to the method of Tamplin et al. (28). After being blocked in PBST supplemented with 10% FCS overnight at 4°C, the membrane was incubated with hybridoma cell culture supernatant for 2 h at room temperature followed by three washing steps in PBST to remove unbound antibodies. Mouse preimmune serum diluted 100-fold in PBS–5% FCS and PBS–5% FCS without supplement were used as negative controls. The blotting membrane was incubated with a peroxidase-conjugated anti-mouse serum diluted 1:1,500 in PBST–10% FCS and 4-chloro-1-naphthol for color development (30).

RESULTS

Development of MAbs. In the primary screening, all supernatants from the hybridomas were tested in ELISAs with living cells of the bacterial isolates *Comamonas acidovorans* PX54, *C. johnsonae* PX62, and *A. hydrophila* PU7718. Only the MAbs which showed strong reactions against the corresponding target strains were then tested by indirect immunofluorescence assays to look for those antibodies which showed strong reactivities against a surface antigen as detected by a ring-like fluorescence of the bacterial cell (ring fluorescence). In order to find MAbs against *Comamonas acidovorans* PX54, we carried out one fusion experiment and found four hybridoma supernatants that showed positive results in the immunofluorescence test. For one of these anti-PX54 antibodies (I4B1), we observed an intense fluorescent labelling of the entire bacterial surface. We performed two fusion experiments with isolate PX62 and detected five hybridoma clones that were positive by ELISA. However, only one supernatant (II1B2) resulted in a ring-like labelling of the target microorganism. We performed one fusion experiment against isolate PU7718 and detected by ELISA only one hybridoma clone which produced an antibody (III4G8) reacting with the bacterial strain used for immunization. This antibody demonstrated also a strong surface label in the immunofluorescence microscopy test. The results of the primary screening for all MAbs positive by ELISA and immunofluorescence testing are summarized in Table 2.

Western blotting experiments. Various Western blotting experiments were done in order to identify of the specific antigens recognized by the MAbs. As shown in Fig. 1A, MAb I4B1, directed against *Comamonas acidovorans* PX54, recognized a double band of LPS in the higher-molecular-weight range.

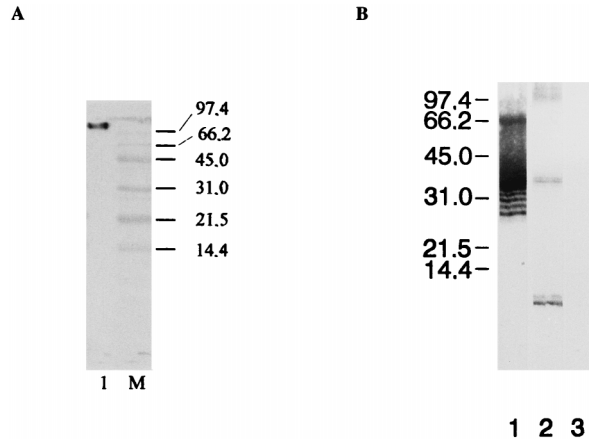

FIG. 1. Western blots of bacterial antigens with the relevant MAbs. (A) LPS preparation from isolate *Comamonas acidovorans* PX54 with MAb I4B1. Lane 1, MAb I4B1; lane M, molecular weight marker for proteins (in thousands). (B) Western blot of the LPS preparation of *A. hydrophila* PU7718 after proteinase K digestion with MAb III4G8. Lane 1, MAb III4G8; lane 2, mouse immune serum anti-PU7718; lane 3, normal mouse serum. Molecular weight markers (in thousands) are noted at the left.

TABLE 3. Cross-reactivities of the MAbs I4B1 (anti-PX54), II2B1 (anti-PX62), and III4G8 (anti-PU7718) with bacterial reference strains[a]

Strain	Cross-reactivity by ELISA (extinction, 490 nm) of MAb[b]:		
	I4B1 (anti-PX54)	II1B2 (anti-PX62)	III4G8 (anti-PU7718)
Paracoccus denitrificans DSM 65^T	0.034	0.040	0.021
Deleya marina DSM 25374	0.021	0.040	0.021
Enterobacter aerogenes DSM 30053^T	0.028	0.048	0.019
Agrobacterium radiobacter DSM 30147^T	0.036	0.059	0.032
Vibrio haloplanktis ATCC 14393	0.033	0.106	0.030
Cytophaga johnsonae DSM 425	0.027	0.046	0.014
Pseudomonas aeruginosa DSM 50071^T	0.032	0.039	0.021
Pseudomonas putida DSM 3931	0.021	0.036	0.038
Vibrio anguillarum ATCC 19264	0.042	0.061	0.021
Alcaligenes faecalis DSM 30030^T	0.024	0.090	0.025
Flavobacterium breve DSM 30096	0.028	0.052	0.019
Alcaligenes xylosoxidans DSM 30026^T	0.041	0.090	0.019
Comamonas acidovorans PX54	**1.942**	0.048	0.018
Cytophaga johnsonae PX62	0.028	**1.883**	0.019
Aeromonas hydrophila PU7718	0.024	0.041	**1.790**
Conjugate control	0.031	0.031	0.026

[a] Bacterial reference strains are from a broad spectrum of genera that are of relevance in aquatic ecosystems as determined by ELISA
[b] Values above background are noted in boldface type.

These bands were detectable only when the purified LPS material was used for SDS-PAGE and Western blot analysis. With a crude bacterial extract of strain PU7718 (*A. hydrophila*), the MAb III4G8 (anti-PU7718) produced a ladder-like banding pattern in the Western blot, indicating that this antibody reacted with a variety of LPS molecules of this gram-negative microorganism (Fig. 1B). Additionally, we tested the mouse hyperimmune serum by Western bloting, which showed, in addition to the ladder-like pattern, a strong reaction in the range of the lipid A structure. For MAb II1B2 (anti-PX62), we could not detect any specific reaction with blotted material from isolate *C. johnsonae* PX62 (data not shown).

Specificity testing of the MAbs. The specificities of the MAbs I4B1, II1B2, and III4G8 were tested against a panel of reference strains, as well as isolates from the same environment (Tables 1 and 3). In a first screening, we tested the reactivities of the MAbs to 12 reference strains belonging to a variety of species that are known for their relevance to aquatic ecosystems and that are phylogenetically diverse. We could not detect any cross-reactivity against these general reference strains by ELISA (Table 3). In addition, we performed specificity tests that included reference strains that were closely related, with respect to their taxonomies, to the isolates or strains that had been isolated either during the mesocosm experiments or directly from lake water (Table 4). We tested the specificity of MAb I4B1 (anti-PX54) against a bacterial strain that is closely related to *Comamonas acidovorans* PX54 as well as against more distantly related microorganisms. We could not find cross-reactivity with this or with any other microorganisms which we used in these tests. The only exceptions were the high level of extinction that occurred when this antibody was tested against strains PX23 and PX41 and a slightly increased level of extinction that occurred against strain PX60. All these strains belonged to the *C. johnsonae* type II group, and we also found positive signals of the conjugate against these strains.

We tested MAb III4G8, generated for the detection of *A. hydrophila* PU7718, against various type strains of the genus *Aeromonas* and also against additional isolates from Lake Plußsee. Neither the *A. hydrophila* type strain nor type strains from other *Aeromonas* species produced a positive signal by ELISA. On the other hand, we detected strong ELISA signals against the isolates PU77-8 and PU69-7 (Table 4), which were isolated from Lake Plußsee at different times and belonged to the *A. hydrophila* complex, as demonstrated by LMW-RNA profiling (Fig. 2). We compared the reaction pattern of MAb III4G8 as determined by Western blot analysis with the fingerprints of the LPS preparation of isolate PU7718 and with those of the LPS preparations from the additional lake isolates PU77-8 and PU69-7, which were recognized by this MAb. Identical fingerprints of both LPS preparations (Fig. 3) indicated that the three isolates, PU7718, PU77-8, and PU69-7, are identical or closely related strains. A weak ELISA signal of MAb III4G8 against isolate PU66-15 was observed (Table 4), but no specific labelling could be detected with this strain by immunofluorescence testing (data not shown). There was no cross-reaction of MAb III4G8 with two other *A. hydrophila* strains (PU84-20 and PU69-8) that had been isolated from the same habitat and, like PU69-8, even from the same water sample as one strain (PU69-7) that reacted with the antibody. This indicates that MAb III4G8 is specific for the three *A. hydrophila* strains, PU7718, PU77-8, and PU69-7, but that it is not specific for other strains from the same species and habitat.

The MAb II1B2 showed no reaction against the *C. johnsonae* type strain and *C. johnsonae* DSM 425, but positive reactions were observed against other *C. johnsonae* strains (e.g., PX60 and PX58) belonging to the *C. johnsonae* type II subgroup and isolated from the same mesocosm (on the same day) from which strain PX62 was isolated. Two of the *C. johnsonae* strains (PX23 and PX41) showed increased reactivity with the conjugate used with the bacteria in the ELISA, so we found strong cross-reactivities of the polyclonal conjugate antibodies with these strains of the *C. johnsonae* complex, and these strains were the only microorganisms which produced positive signals when we tested the reactivities of MAbs I4B1 (anti-PX54) (Table 4).

Detection of specific bacteria in lake water with MAbs. Water samples along a depth profile were taken from Lake Plußsee approximately 3 years after the isolation of the relevant strains. After immunological staining of the bacterioplankton with the MAbs I4B1 (anti-PX54) and III4G8 (anti-PU7718), we estimated the total number of detectable bacterial cells in each depth sample. We counted only those cells showing a clear and distinct ring fluorescence after immunological stain-

TABLE 4. Cross-reactivities of the MAbs I4B1, II1B2, and III4G8 with different bacterial isolates from Lake Plußsee and closely related reference strains by ELISA

Strain	Cross-reactivity by ELISA (extinction, 490 nm) of MAb[a]:			
	I4B1 (anti-PX54)	II1B2 (anti-PX62)	III4G8 (anti-PU7718)	Conjugate
Comamonas acidovorans PX54	**2.240**	0.046	0.055	0.024
C. johnsonae (type II) PX62	0.020	**2.019**	0.062	0.024
C. johnsonae (type I) PX3	0.033	0.034	ND	0.040
C. johnsonae (type I) PX20	0.031	0.044	ND	0.060
C. johnsonae (type II) PX60	0.328	**2.678**	ND	0.238
C. johnsonae (type II) PX41	**1.768**	**1.754**	ND	**1.709**
C. johnsonae (type II) PX58	0.230	**3.000**	ND	0.197
C. johnsonae (type II) PX51	0.022	0.059	ND	0.024
C. johnsonae (type II) PX23	**2.478**	**2.556**	ND	**2.438**
C. johnsonae (type II) PX43	0.129	0.173	0.158	0.140
C. johnsonae (type II) PX25	0.143	0.207	0.160	0.136
C. johnsonae (type II) PX28	0.168	0.257	0.175	0.228
C. johnsonae (type II) PX46	0.097	0.188	0.104	0.140
C. johnsonae (type II) PX65	0.055	0.084	0.078	0.034
C. johnsonae DSM 2064^T	0.151	0.054	ND	0.047
C. johnsonae DSM 425	0.027	0.046	ND	0.075
A. hydrophila PU7718	0.020	0.115	**2.146**	0.024
A. hydrophila PU77-8	0.129	0.152	**2.440**	0.100
A. hydrophila PU69-7	0.132	0.148	**2.233**	0.110
A. hydrophila PU69-8	0.025	0.142	0.056	0.023
A. hydrophila PU84-20	0.097	0.118	0.113	0.065
A. hydrophila PU84-24	0.092	0.113	0.092	0.055
A. hydrophila subsp. *hydrophila* DSM 30187^T	0.066	0.168	0.073	0.027
A. hydrophila subsp. *androgena* DSM 30188^T	0.099	0.110	0.048	0.010
A. hydrophila subsp. *proteolytica* DSM 30189^T	0.115	0.139	0.055	0.009
A. punctata DSM 30190	0.129	0.102	0.108	0.073
A. media DSM 4881^T	0.118	0.110	0.052	0.080
A. schubertii DSM 4882^T	0.117	0.129	0.054	0.010
PU66-15 (related to PX54)	0.105	0.101	0.310	0.063

[a] ND, not determined. Values above background are noted in boldface type.

ing. Figure 4 presents an overview of the cell numbers of the isolates PX54 (*Comamonas acidovorans*) and PU7718 (*A. hydrophila*) from different depths of Lake Plußsee in the spring of 1993. These data were compared with the total numbers of bacteria at each depth. The maximum total number of bacteria was observed at a depth of 5 m, with bacterial numbers in the range of 19.8×10^6 and 12.9×10^6 cells/ml in the upper 10 m. From 15 m downwards, the total number of bacteria decreased from 8.9×10^6 to 5.7×10^6 cells/ml. As determined by immunofluorescence microscopy with the MAb III4G8, we detected cells of *A. hydrophila* PU7718 predominantly at the surface of the lake down to 10 m, with a maximum of 8×10^3 cells/ml at the 6-m depth. Below 10 m, the abundance of *A. hydrophila* PU7718 decreased by a factor of 20. Cells of *Comamonas acidovorans* PX54 showed a rather different depth distribution for the upper 10 m, with a maximum cell count observed at the surface (0 m). Below 10 m, the cell counts for *Comamonas acidovorans* PX54 dropped to the detection limit (Fig. 4).

In the lake water samples, *A. hydrophila* was observed mostly as single cells (Fig. 5A) and sometimes as double cells, whereas *Comamonas acidovorans* cells were seen mostly as clusters of 10 to 50 cells (Fig. 5B) and only very rarely as single bacterial cells.

DISCUSSION

Screening for strain-specific MAbs for environmental applications. Despite the great analytical potential of MAbs, their application as specific probes for the detection of bacteria in the environment is difficult because of their strong cross-reactivities with bacterial surface structures. Due to this cross-reactivity, it is not easy to select MAbs that show strong reactivities to target bacterial strains and that do not react with any other bacterial strain. Therefore, we have developed a screening strategy for the development of strain-specific MAbs that includes other isolates of the same species from the same habitat.

For the primary screening test to select MAbs with high levels of specificity for surface epitopes of bacterial strains, we favored the strategy of testing all hybrid supernatants from each fusion experiment not only against the immunizing bacterial strain but also against the other two isolated microorganisms. For each fusion experiment, we detected a relatively high number of primary hybrid cultures (more than 50% of the wells) but there was only a very small number of primary cultures (about 5% of the total number of hybridoma culture plate cavities) which reacted only with the homologous strain by ELISA. Comparable to this data, we also found strong cross-reactivities of the polyclonal antisera raised against these three bacterial isolates in mice used for the production of the MAbs. Each antiserum reacted not only with the homologous bacterial strain used for immunization but also with the two other microorganisms with high titers. In addition to the possibility of a high number of cross-reacting antibodies produced by the primary hybrid cultures, a second reason for this surprising result is the relatively high number of cavities of the hybridoma culture plates, which showed more than one hybrid cell clone. If many of the primary cell clones produced MAbs, the probability that different antibodies, with different specificities, were secreted in the cell culture supernatant of a single culture plate well is relatively high. On the other hand, it is

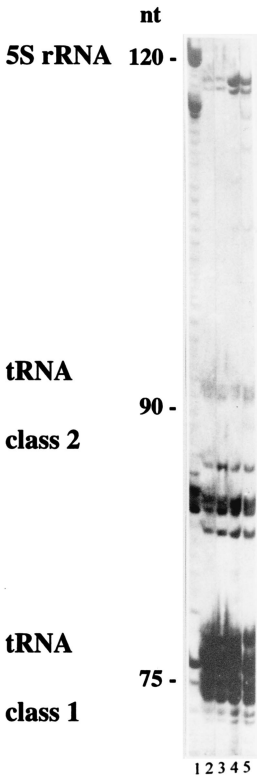

FIG. 2. LMW-RNA (5S rRNA and tRNA) profiles of *A. hydrophila* isolates and the type strain. Lane 1, molecular size markers (hydrolyzed 5S rRNA, $tRNA_{phe}$, and $tRNA_{tyr}$ from *Escherichia coli*); lane 2, *A. hydrophila* PU69-7; lane 3, *A. hydrophila* PU77-8; lane 4, *A. hydrophila* PU7718; lane 5, *A. hydrophila* DSM 30187^T.

possible that during the immobilization of the living bacteria on the microtiter plate, part of the bacteria were disrupted and cytoplasmatic molecules or their breakdown products were attached to the surfaces of the microtiter wells. Thus, antigenic structures, which created B-cell clones in mice during immunization, can increase the number of hybrid cell clones producing cross-reacting MAbs.

For the successful use of MAbs against isolated bacterial strains in the natural environment, it was important to exclude

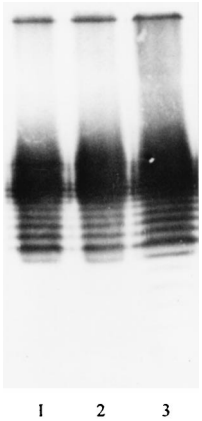

FIG. 3. Western blot of LPS preparations of three different *A. hydrophila* isolates after proteinase K digestion with MAb III4G8 (anti-PU7718). Lane 1, *A. hydrophila* PU69-7; lane 2, *A. hydrophila* PU77-8; lane 3, *A. hydrophila* PU7718.

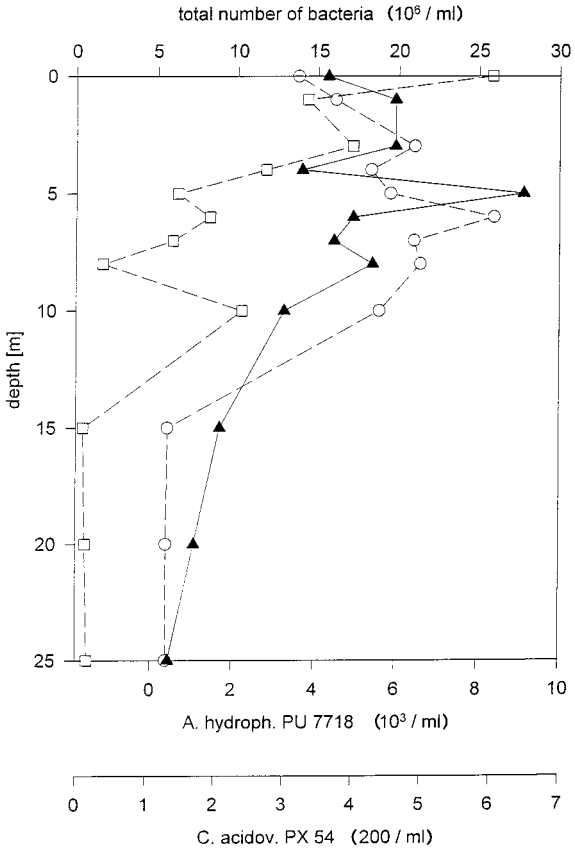

FIG. 4. Depth profile based on bacterial numbers in samples from Lake Plußsee collected on 19 April 1993. Filled triangles, total number of bacteria; open circles, specific cell counts for *A. hydrophila* (A. hydroph.) PU7718; open squares, specific cell counts for *Comamonas acidovorans* (C. acidov.) PX54.

cross-reactivity with other microorganisms that are of relevance in the habitat of interest. Therefore, we tested the reactivities of the MAbs against bacterial isolates belonging to the same taxonomic groups as bacteria existing in the investigated environment (Lake Plußsee). The results of the ELISA experiments for the estimation of cross-reactions of the MAbs selected during immunofluorescence tests (I4B1, II1B2, and III4G8) showed no positive signals against bacteria that are of relevance in the ecosystem from which the bacterial strains *Comamonas acidovorans* PX54, *C. johnsonae* PX62, and *A. hydrophila* PU7718 had been isolated (Tables 3 and 4). In addition to the relative abundance of strains of *C. johnsonae* and *A. hydrophila* among heterotrophic isolates from lake water, the relevance of these two species in Lake Plußsee water has also been demonstrated by direct analysis of LMW RNA from bacterioplankton (15).

A second important point was to demonstrate the strain specificities of the MAbs when they were tested against the immunizing isolate and other strains of the same bacterial species. In contrast to the results produced with reference strains, we observed strong reactions of the antibodies II1B2 (anti-*C. johnsonae* PX62) and III4G8 (anti-*A. hydrophila* PU7718) against strains that had been isolated from the same ecosystem and that were closely related to the strains used for the generation of the MAbs (Table 4). For the two *A. hydrophila* isolates that react with the anti-PU7718 MAb, PU77-8 and PU69-7, one strain (PU77-8) had been obtained from the

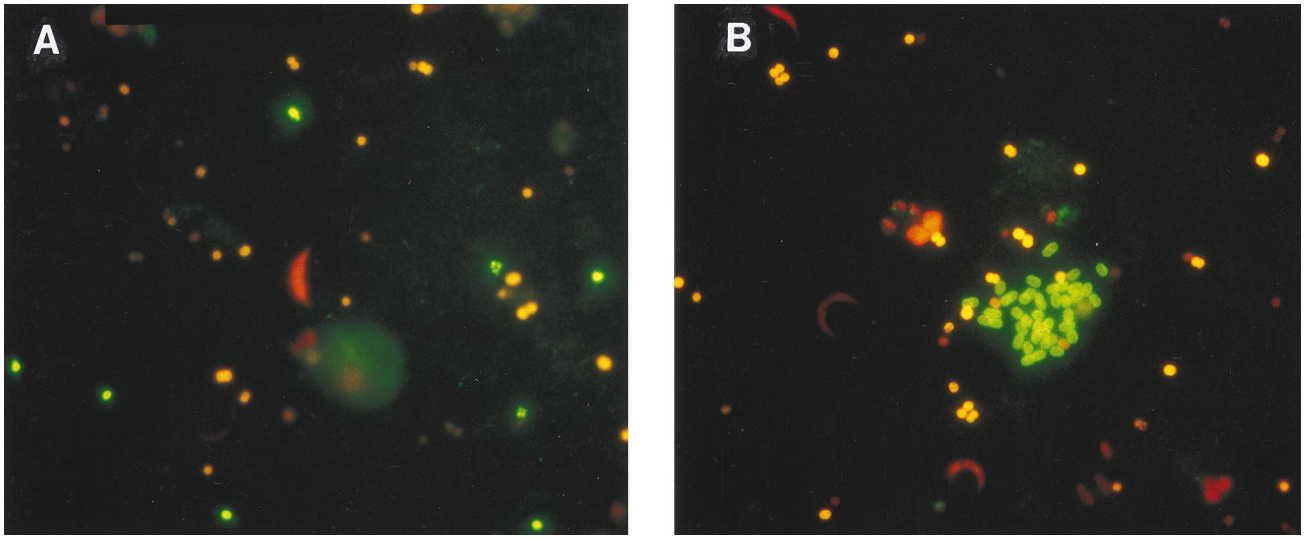

FIG. 5. Representative photomicrographs of immunofluorescence staining of bacterioplankton in samples from the depth profile of Lake Plußsee collected on 19 April 1993 with two different MAbs. (A) MAb III4G8 for the detection of *A. hydrophila* PU7718; (B) MAb I4B1 for the detection of *C. acidovorans* PX54. Positive cells show green fluorescence from the DTAF of the secondary antibody. Yellow and orange fluorescence stems from autofluorescence of chlorophyll *a*, indicating indigenous cyanobacteria or eukaryotic algae.

same water sample and the other (PU69-7) had been obtained 2 months earlier. As the molecular identification data in Fig. 2 and 3 demonstrate, these isolates are indistinguishable and may be coisolates of the same clone as PU7718. On the other hand, the three additional *A. hydrophila* isolates, as well as all reference strains of *A. hydrophila*, showed no positive reaction with MAb II4G8, thereby indicating the high specificity of the developed MAb. More difficult to explain was the reaction of MAb II1B2 in the ELISA tests against *C. johnsonae* isolates which were isolated during the mesocosm experiments. We have observed in these experiments positive reactions against two *C. johnsonae* type II isolates (*C. johnsonae* PX60 and *C. johnsonae* PX58), both of which were isolated at day 16 of the mesocosm experiment from mesocosm 8 (Table 1). With two isolates (*C. johnsonae* PX41 and *C. johnsonae* PX23) we detected strong reactions of the antibody conjugate used with these bacterial isolates.

With MAb I4B1 (anti-PX54), we found a weak double band in the immunoblot test only when we used a purified LPS preparation of *Comamonas acidovorans* PX54. Therefore, it is possible that the expression of LPS on the bacterial surface is low and thus that the amount of LPS loaded on the gel was too low to generate a detectable signal. Another explanation may be that the recognized epitope is unstable under these conditions.

Application of strain-specific MAbs in lake water samples. For the successful use of MAbs for the identification and quantification of bacterial cells in natural water samples, not only must the antibodies show high reactivities by the immunofluorescence technique but also their signals should be well discriminated from unspecific background fluorescence. Commonly used techniques for the enumeration of microorganisms in water samples comprise filtration on a polycarbonate membrane, staining of the bacteria by a DNA-specific dye, e.g., DAPI, and use of specific immuno-probes followed by microscopic examination (4). In freshwater samples we have observed, in many cases, high concentrations of particles containing fluorescent material that sometimes make it difficult to distinguish between specifically labelled bacteria and background fluorescence or autofluorescent material such as cyanobacteria. For that reason we favored antibodies showing not only a strong label against the homologous strain in the immunofluorescence test but also a typical ring fluorescence, which indicates that the bacterial epitope recognized by the antibody is located on the surface of the microorganism and occurs there at a high concentration. With such antibodies for identification of bacterial cells in environmental samples, the differentiation of the specifically labelled cells from other fluorescent material is easier to determine.

We performed immunofluorescence microscopy with a variety of freshwater samples with all of the antibodies listed in Table 2. We observed a clear labelling of single bacterial cells in environmental samples only for antibodies I4B1 (anti-PX54) (Fig. 5B) and III4B8 (anti-PU7718) (Fig. 5A). With MAb II1B2 (anti-PX62), a high number of fluorescently labelled, very small particles and strongly labelled, hazy material could be seen but no definitively labelled bacterial cells were observed. In contrast to what occurred in the MAb reaction, no labelled material was observed in the negative controls (conjugate negative control or an MAb with another specificity). For that reason, it is conceivable that the antibody recognized the homologous epitope which is present in slime at high concentrations. It is well known for members of the genus *Cytophaga*, such as isolate PX62 (*C. johnsonae*), that many species produce high amounts of extracellular polysaccharides. This slimy material is able to cover the bacterial cell surface recognized by the specific antibodies, such that the antibody cannot bind to the epitope. We also selected other hybridomas producing specific MAbs against isolate PX62. However, all selected MAbs detected the bacteria, by immunofluorescence assays, only when the cells formed aggregates. All MAbs did not react with single bacterial cells of the same isolate (data not shown).

Using the MAbs I4B1 and III4G8, we determined the number of bacterial cells of the isolates PX54 and PU7718 in freshwater samples of a depth profile of Lake Plußsee from April 1993 (Fig. 4). The distribution of isolate PU7718 was observed over the depth profile corresponding to the different

concentrations of oxygen at different levels of depth. The predominant abundances of all PU7718 bacteria were detected in the range between 0 and 10 m, where higher concentrations of oxygen, as well as higher temperatures, were measured. The strong decrease in cell numbers of PU7718 below 10 m, by more than an order of magnitude, does not correspond with the total numbers of bacteria. This lack of correspondence indicates that the *A. hydrophila* cells occur primarily in the epi- and metalimnion and are present in background numbers only in the hypolimnion. In comparison with isolate PU7718, we observed a much lower number of PX54 cells at each depth. Additionally, a much lower decrease in the cell numbers was observed with increasing depth and with decreasing oxygen concentration and temperature.

The environmental detection of two strains of heterotrophic bacteria (*A. hydrophila* PU7718 and *C. acidovorans* PX54) of different genera, 3 years after their initial isolations in the same habitat, indicates that there is stability over time in the structure of the heterotrophic bacterial community at the strain level. Similar observations for a marine pelagic environment were made recently by randomly amplified polymorphic DNA fingerprinting for a set of *Shewanella putrefaciens* isolates (34). On the other hand, the specific cell numbers observed in situ for the two strains were only as high as half a thousandth part of the total cell numbers. These numbers are in good agreement with the numbers of ammonium-oxidizing cells in the same ecosystem or other pelagic habitats for various heterotrophic bacteria, as determined with polyclonal antibodies (31, 32). However, these numbers are in contrast to the LMW-RNA determinations made 3 years earlier, when at least *A. hydrophila* was detected at a relative abundance of a few percent, with respect to the direct analysis of 5S rRNA (15). The discrepancy of approximately 2 orders of magnitude between results of the two methods for the in situ abundance of PU7718 may be due to various reasons: (i) 5S rRNA abundance was also low in the samples from April 1993, (ii) 5S rRNA abundance strongly overestimated cellular abundance, and (iii) the specificities of the MAbs were far too high to detect all cells of the species. A direct comparison of different methodologies, with the same samples, is necessary for a better understanding of the specificities of these molecular approaches for the direct determination, at the single-cell level, of the levels of abundance of bacterial species in natural ecosystems (2). These other methodologies should comprise different types of molecular probes, such as rRNA-targeted oligonucleotides and MAbs generated through westprinting and with chromosomal paints, with different levels of resolution, i.e., to the levels of strain, species, and genus (1, 20, 29). Furthermore, to understand the detailed specificities of MAbs for in situ detection, the analysis of more isolates of the same species and from the same habitat is necessary. With the described MAbs, these isolates can now be enriched by immunocapture techniques and rapidly detected by Western blotting of colonies growing on agar plates (5, 9).

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