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Fluorescently Labeled Virus Probes Show that Natural Virus Populations Can Control the Structure of Marine Microbial Communities†

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Fluorescently stained viruses were used as probes to label, identify, and enumerate specific strains of bacteria and cyanobacteria in mixed microbial assemblages. Several marine virus isolates were fluorescently stained with YOYO-1 or POPO-1 (Molecular Probes, Inc.) and added to seawater samples that contained natural microbial communities. Cells to which the stained viruses adsorbed were easily distinguished from nonhost cells; typically, there was undetectable binding of stained viruses to natural microbial assemblages containing $>10^6$ bacteria ml^{-1} but to which host cells were not added. Host cells that were added to natural seawater were quantified with 99% ± 2% (mean ± range) efficiency with fluorescently labeled virus probes (FLVPs). A marine bacterial isolate (strain PWH3a), tentatively identified as *Vibrio natriegens*, was introduced into natural microbial communities that were either supplemented with nutrients or untreated, and changes in the abundance of the isolate were monitored with FLVPs. Simultaneously, the concentrations of viruses that infected strain PWH3a were monitored by plaque assay. Following the addition of PWH3a, the concentration of viruses infecting this strain increased from undetectable levels (<1 ml^{-1}) to 2.9×10^7 and 8.3×10^8 ml^{-1} for the untreated and nutrient-enriched samples, respectively. The increase in viruses was associated with a collapse in populations of strain PWH3a from ca. 30 to 2% and 43 to 0.01% of the microbial communities in untreated and nutrient-enriched samples, respectively. These results clearly demonstrate that FLVPs can be used to identify and quantify specific groups of bacteria in mixed microbial communities. The data show as well that viruses which are present at low abundances in natural aquatic viral communities can control microbial community structure.

There are many instances in which it is necessary to quantify the concentrations of specific types of bacteria in aquatic samples. These instances include monitoring the abundance of bacterial pathogens for the purpose of public health and monitoring the dynamics of particular subsets of the bacterial community in ecological studies. Traditionally, the abundances of specific microorganisms in natural waters have been monitored by culturing each organism of interest on selective medium, although in recent years a number of other approaches have emerged, including the use of fluorescently labeled oligonucleotides (1, 11) and antibodies (7, 26). However, low cellular concentrations of ribosomes, variability in the specificity and intensity of antibody labeling, and high levels of background fluorescence can interfere with these methods in some applications (6, 10). Moreover, the specificities of oligonucleotide and antibody probes, although excellent for some applications, are less suited for others. For example, it is not clear whether oligonucleotides or antibodies have specificities that are appropriate for discriminating among populations of cells that can be infected by viruses.

Viruses are extremely abundant and biologically active members of marine and freshwater microbial communities, in which they can be responsible for a considerable portion of the total mortalities of bacteria and cyanobacteria (12, 22). Despite the abundance and biological importance of viruses, their roles in controlling microbial-community structure and in driving microbial-population dynamics are unclear. In some cases the abundances of viruses and hosts appear to be positively correlated (9, 19, 24, 27), while in other instances viruses appear to cause the apparent lysis of entire populations within the microbial community (3, 4). It has been difficult to ascertain the effect of viruses on microbial-community structure, in part because the abundance of cells which could be infected by specific viruses could not be reliably estimated.

Phage typing, or the use of viruses to identify and differentiate among bacterial strains, is a sensitive procedure that can be used to characterize clinical and environmental isolates (8, 29). In this study we developed a protocol with fluorescently labeled viruses as probes to identify and enumerate specific strains of heterotrophic bacteria and cyanobacteria that were added to natural marine microbial communities. We applied this approach, in conjunction with traditional plaque assays, to monitor the dynamics in situ of a marine bacterium and the lytic viruses which infect it. These results clearly demonstrate that viruses which are present in seawater, even at concentrations of <1 infective unit ml^{-1}, can control marine microbial-community composition.

MATERIALS AND METHODS

Virus isolates and hosts. The cyanophages (S-BBS1 [family, *Syphoviridae*] and S-PWM2, S-PWM3, and S-PWM4 [*Myoviridae*]) and bacteriophages (PWH3a-P1, LB1VM-P1, and LMG1-P4 [*Myoviridae*] and LB1VL-P1b [*Podoviridae*]) that were used in this study were isolated from the coastal waters of Texas and were amplified in culture with their respective hosts (23, 25). Following lysis, the cultures were filtered through 0.2-μm-pore-size Durapore membranes to remove cellular debris and were stored in the dark at 4°C.

Preparation of fluorescently labeled viruses. Aliquots (1.4 ml) of culture lysate

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were transferred to 1.5-ml microcentrifuge tubes and sealed with O-ring screw caps. The microcentrifuge tubes were suspended in ultracentrifuge tubes (14 by 95 mm) filled with water and then spun at 155,000 × *g* for 1 h. The microcentrifuge tubes were floated out of the ultracentrifuge tubes by adding water, and the supernatant was carefully withdrawn, except for 20 μl which was used to gently resuspend the pellet. Each of the virus isolates was stained with about 1 μl of staining solution (1 mM YOYO-1 or POPO-1 in dimethyl sulfoxide-H_2O [1:4]; Molecular Probes, Inc., Eugene, Oreg.) per 10^{11} viruses, except for strain PWH3a-P1, which was stained with a saturated solution of POPO-1 (0.5 mg in 100 μl of deionized distilled water and then subjected to sonication for 15 min). The phages were incubated for 2 days in the dark at 4°C, washed twice in culture medium, pelleted as described above, and stored in the dark at 4°C.

Sampling sites. Seawater samples were taken from Texas coastal waters at the following locations: Gulf of Mexico (27°31′N, 96°18′W), Aransas Pass (27°50′N, 97°02′W), Laguna Madre (27°30′N, 97°18′W), and the boat harbor of the University of Texas Marine Science Institute. Water for the microcosm studies was collected from Aransas Pass from the pier at the Marine Science Institute and filtered at low pressure (10 kPa) through 1-μm-pore-size polycarbonate filters to remove bacterial grazers. Samples were stored for <1 h in the dark at 4°C until the beginning of the microcosm experiments.

Bacterial and viral counts. Epifluorescence microscopy was used to count bacteria stained with DAPI (4′6-diamidino-2-phenylindole), which specifically stains double-stranded DNA (20). The net bacterial growth rate was approximated as the average increase in bacterial numbers between two measurements. Viruses were counted on ceramic 0.02-μm-pore-size Anodisc 25 membranes (Whatman, Inc.) after staining with Yo-Pro-1 (4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylmethyledene]-1-(3′-trimethylammoniumpropyl)-quinolinium diiodide) (Molecular Probes) following the protocol of Hennes and Suttle (15). The abundance of phages infecting the marine bacterium PWH3a (tentatively identified as *Vibrio natriegens*) was determined by plaque assay (25).

Tagging of cells with fluorescently labeled virus probes (FLVPs). Approximately 10^8 fluorescently labeled viruses ml^{-1} of water were added to natural water samples in which biological activity had been stopped by the addition of 2 mM NaCN. In some cases the natural microbial communities were supplemented with up to 6×10^5 host cells ml^{-1} from cultures. After incubating for 10 to 60 min, a 1-ml subsample was filtered onto a 0.2-μm-pore-size Anodisc 25 membrane overlaid on a 0.45-μm-pore-size nitrocellulose filter. The Anodisc membrane was laid on a glass slide, and a drop of culture medium, particle-free seawater, or a mixture of 40% glycerol in water (samples probed with PWH3a-P1) was placed on the filter and covered with a coverslip. The slides were viewed at a magnification of ×1,000 with an Olympus IMT-2 epifluorescence microscope equipped with either an acridine orange filter set for YOYO-1-labeled viruses (excitation wavelength, 490 nm; dichroic mirror, 500 nm; barrier filter, 515 nm) or a violet filter set for POPO-1- and YOYO-1-labeled viruses (excitation wavelength, 405 nm; dichroic mirror, 455 nm; barrier filter, 455 nm). More than 200 tagged cells were counted in a minimum of 20 random fields for each of the triplicate samples. The abundance of tagged cells was considered to be below the detection limit if no tagged cells were observed in 50 fields of view (i.e., <200 cells ml^{-1}).

The accuracy of using FLVPs to estimate the abundance of host cells was tested by adding known numbers of host cells to natural seawater samples (ca. 10% of total bacteria) and then estimating the abundance of host cells in these samples by the FLVP method. The abundance of tagged cells was compared with that in controls to which host cells were not added.

Microcosm experiments. A seawater sample was filtered through 1-μm-pore-size polycarbonate membranes to remove grazers and was divided into 500-ml aliquots and transferred to six loosely capped polyethylene bottles. Three bottles were enriched with 5 mg of Casamino Acids $liter^{-1}$ and 5 mg of peptone $liter^{-1}$ (1% CPM) at time zero, and 2.8 h later all of the bottles were inoculated with a marine bacterium (strain PWH3a) which was originally isolated from the sampling site (25). The inocula were taken from an early stationary batch culture and were added so that they constituted approximately 20% of the total number of bacteria in the samples. The microcosms were incubated at room temperature in the dark and gently mixed on a shaker at 150 rpm. At intervals of 3 to 12 h, samples were taken for determining the total abundances of free viruses and bacteria, viruses infecting strain PWH3a, and cells that were tagged by FLVPs made from the virus PWH3a-P1.

RESULTS AND DISCUSSION

FLVPs. The recent advent of brightly fluorescent cyanine-based dyes that have very high binding coefficients for nucleic acids (16) and that can be used to stain and visualize individual viruses by epifluorescence microscopy (15) suggested that fluorescently labeled viruses with well-defined host ranges could be used to tag and identify specific types of bacteria within mixed microbial communities. We tested this by staining a number of different viruses which infect marine heterotrophic bacteria (strains PWH3a, LMG1, and LB1) (25) and cyanobacteria (*Synechococcus* sp. strains DC2, SYN48, SNC1, and SNC2) (23) with the dye YOYO-1 or POPO-1. The stained viruses were added to host cell cultures and to seawater samples containing natural microbial communities plus added host cells. FLVPs did not adsorb to nonhost cells, and tagged cells were easily distinguished from detritus or other particulate material to which FLVPs attached (Fig. 1). Moreover, of the host systems that we had in culture, the FLVPs attached only to cells in which the unstained viruses could replicate. Although some viruses can attach to cells in which they cannot replicate, this did not occur with any of the phage systems that we examined.

There was undetectable binding of fluorescently labeled viruses to natural microbial assemblages containing $>10^6$ bacteria ml^{-1} but to which host cells were not added (Table 1). The only exception was for an FLVP that was made with a cyanophage (S-PWM3) with a relatively broad host range (23); this FLVP attached to about 3% of the *Synechococcus* cells in two samples from the Gulf of Mexico (data not shown). Although the abundance of cells in natural communities that were tagged with the FLVPs was typically below detection limits, host cells that were added to these communities were accurately quantified.

Viral control of microbial-community structure. As we were able to quantify the abundances of specific bacterial strains in mixed microbial assemblages, this allowed us to monitor the population dynamics of a marine bacterium that was introduced into a natural marine microbial community. Moreover, by simultaneously monitoring the abundances of viruses that infect strain PWH3a we were able to determine if natural marine virus assemblages can regulate marine microbial community structure. Viruses are recognized as being an abundant, dynamic, and ecologically significant component of aquatic ecosystems (2, 5, 12), and estimates suggest that, on average, about 20% of marine bacteria are lysed by viruses on a daily basis (22); hence, viruses could be important elements structuring microbial communities. Determining whether viruses affect the structure of microbial communities has been hampered, in part, by the difficulty in quantifying the abundances of specific host organisms in natural waters.

The total abundances of bacteria and viruses at the beginning of the experiment were approximately 6×10^6 and 1×10^8 ml^{-1}, respectively, although bacteria that were tagged by the fluorescently labeled virus PWH3a-P1 and viruses infecting bacterial strain PWH3a were undetectable (Fig. 2). Following the addition of strain PWH3a, the abundance of bacteria and the proportion of the community made up of PWH3a continued to increase. In the nutrient-enriched samples, this was followed by a strong increase in viral abundance and a concomitant decrease in bacterial abundance, consistent with viral replication at the expense of bacteria. The peak in the relative abundance of strain PWH3a rapidly decreased from 43.4% of the total bacteria 10 h after the start of the experiment to 0.01% of the community 12 h later. This sudden decrease coincided with the appearance of lytic viruses which infected PWH3a. In the unenriched microcosms, there was also a decrease in PWH3a that was associated with the appearance of lytic viruses, although this did not occur until 32 h after the start of the experiment.

There were two factors that likely contributed to the accelerated collapse of the PWH3a populations in the nutrient-enriched microcosms. Nutrient addition initially resulted in net growth rates for PWH3a that were about five times higher than those in unenriched treatments (1.09 h^{-1} versus 0.23 h^{-1}). Cell yields were also about five times higher in enriched cultures ($[9.5 \pm 1.2] \times 10^6$ and $[2.0 \pm 0.3] \times 10^6$ ml^{-1} [means ± standard deviations] in enriched and unenriched cultures, re-

FIG. 1. Bacteria tagged with FLVPs. (A) Marine heterotrophic bacterial strain PWH3a tagged with phage isolate PWH3a-P1, which was fluorescently labeled with POPO-1 (blue fluorescence). (B) A natural marine bacterial community which includes cyanobacteria of the genus *Synechococcus* (red autofluorescence) as well as marine *Synechococcus* sp. isolate BBC1. *Synechococcus* isolate BBC1 has been tagged with the POPO-1-labeled cyanophage S-BBS1 (blue fluorescent halo). (C) A natural marine bacterial community to which *Synechococcus* sp. isolate BBC1 and a heterotrophic bacterium (isolate PWH3a) have been added. BBC1 has been tagged with the cyanophage S-BBS1 (blue fluorescent halo), and PWH3a has been tagged with phage isolate PWH3a-P1, which was fluorescently labeled with YOYO-1 (green fluorescence). The samples were viewed by epifluorescence microscopy with a blue (A) or violet (B and C) filter set, as described in the text. Pictures were taken at ×1,000 magnification (10- to 30-s exposures; Kodachrome 400 film). Scale bars, 10 μm.

TABLE 1. Accuracy of FLVPs in quantifying bacteria added to natural microbial communities[a]

Host strain[b]	Sample date	DAPI count (mean ± SD) for[c]:		FLVP count for hosts recovered[c] (10^6/ml)	Counting accuracy (%)
		Total bacteria[d] (10^6/ml)	Hosts added (10^6/ml)		
PWH3a	25 Jan. 1994	4.64 ± 0.58	0.52 ± 0.02	0.54 ± 0.01	97
LB1	21 Mar. 1994	3.93 ± 0.12	0.56 ± 0.04	0.55 ± 0.02	101
BBC1	10 May 1994	2.33 ± 0.13	0.061 ± 0.002	0.061 ± 0.004	99

[a] There was undetectable binding of FLVPs to bacteria in seawater controls.
[b] BBC1 is a cyanobacterium (*Synechococcus* sp.); PWH3a and LB1 are marine heterotropic bacteria.
[c] Determined from triplicate samples.
[d] Total bacteria are the sum of natural bacteria and hosts added.

spectively). As the frequency with which viruses contacted PWH3a is proportional to the product of the host and virus concentrations (for examples, see references 9 and 18), the infection rate would also have been about five times higher in the enriched cultures. Moreover, as the number of viruses produced per lytic event increases and the length of the lytic cycle decreases with the growth rate of the host (13, 21), the production rate of viruses and the collapse of the host cell populations were much more rapid in the nutrient-enriched than in the unenriched microcosms (Fig. 2). Consequently, after 46 h viruses infecting PWH3a made up 73 and 8% of the total virus community in the enriched and unenriched treatments, respectively, while the abundances of strain PWH3a decreased to 0.01 and 3% of the total bacteria in the enriched and unenriched treatments, respectively (Fig. 2).

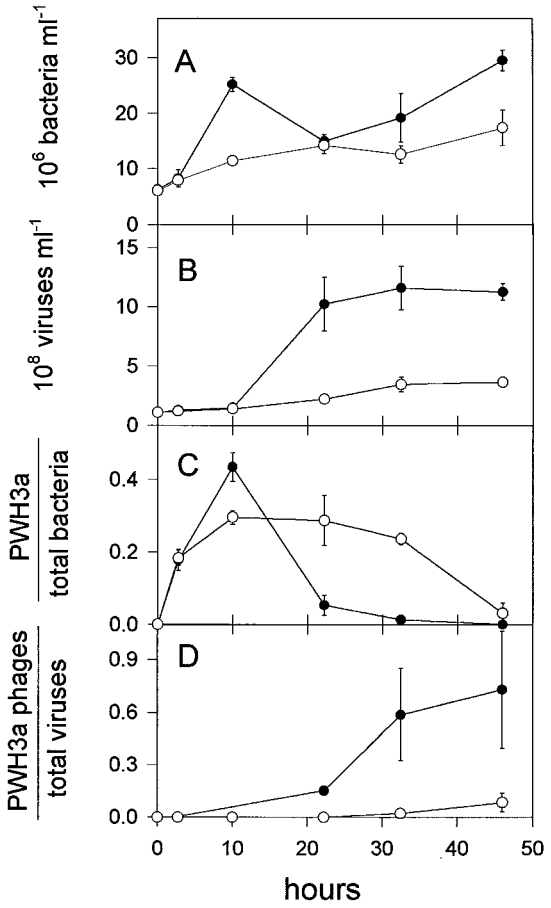

FIG. 2. The total abundances of bacteria (A) and viruses (B) in the microcosm experiments as well as the proportions of the entire bacterial and viral communities that were made up of the marine bacterial isolate PWH3a and the viruses which infect PWH3a (C and D, respectively) are shown. No viruses were added, but at 2.8 h the bacterial isolate PWH3a was added to each microcosm, so that it constituted approximately 20% of the total bacteria. Open circles represent unenriched microcosms, while solid symbols represent microcosms to which peptone and Casamino Acids were added (see text for details). Error bars indicate the standard deviations of the means of data from triplicate microcosms.

Although it is clear that marine viruses are responsible for considerable microbial mortality, it has been difficult demonstrating that viruses regulate the abundances of specific populations of bacteria in aquatic systems and thereby affect microbial-community structure. In some studies, increased virus concentrations have been associated with decreases in the abundance of bacteria or phytoplankton (4, 14, 28); however, overall the highest virus concentrations typically occur when bacteria and phytoplankton are most abundant (9, 17, 19, 24, 27). One reason why it has been difficult to demonstrate that viruses affect the structure of microbial communities is because of the problem of distinguishing subsets of the bacterial community that are susceptible to infection by a specific virus or group of viruses. In this study, we were able to use FLVPs to identify subsets of the microbial community that could be infected by a group of naturally occurring marine viruses and thus were able to demonstrate that these viruses were able to control the abundances of bacteria to which they could attach. As it is possible to label viruses which infect photosynthetic protists (15), it may also be possible to use FLVPs to monitor the dynamics of subsets of the eukaryotic phytoplankton community.

The use of fluorescently labeled viruses with well-defined host ranges should also be appropriate for identifying and quantifying bacteria in clinical applications. For example, phage typing has long been used for the identification of *Salmonella* strains (8, 29), but the protocol requires culturing the bacterium of interest. In contrast, the use of FLVPs should allow the identification and enumeration of *Salmonella* strains without the need of culturing. Moreover, it should be possible to couple the protocol with flow cytometry to increase the sensitivity of detection. The principle limitation of using FLVPs to tag and identify particular subsets of microbial communities will be isolating and identifying viruses that have suitable host ranges. However, even if restricted to those viruses whose host ranges have already been well characterized, the protocol should have the potential for wide application.

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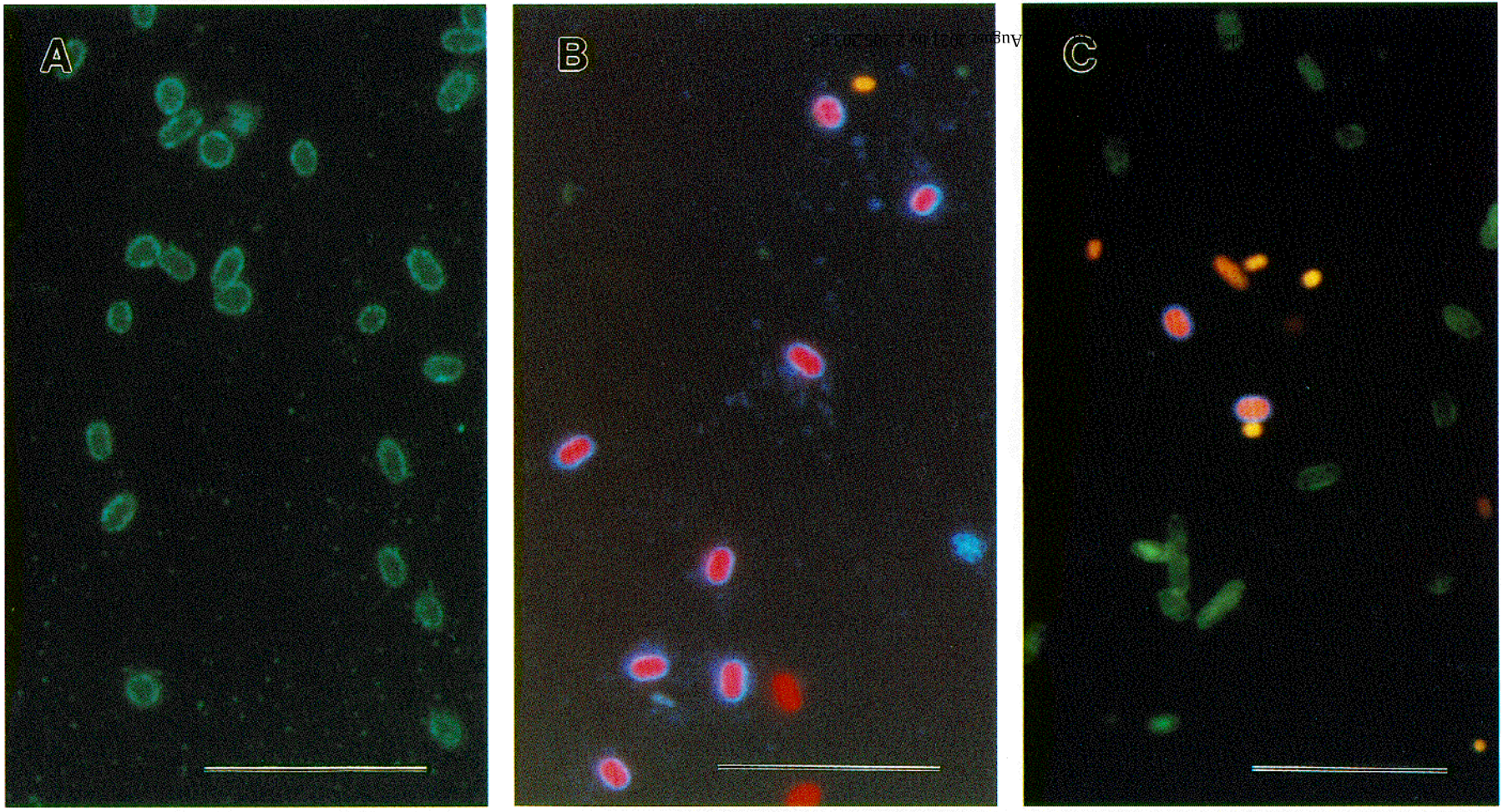
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