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In situ hybridization with rRNA targeted probes as a new tool for the detection of black yeasts and meristematic fungi

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Abstract: *In situ* hybridization with rRNA targeted oligonucleotide probes is presented as a tool for the detection of fungi on material surfaces. A detailed protocol is described as a basis for different applications. The method is especially adapted for meristematic fungi and black yeasts. The entry of the probe through thick and melanized cell walls can be achieved by β-glucanase treatment. As an example the method is applied to fungal biofilms grown on glass and plastics. The potential and the difficulties of the method as for example the autofluorescence of fungi and the limited availability of DNA sequences for the design of taxon specific probes are discussed.

Key words: *in situ* hybridization, small ribosomal subunit, fluorescent probe, fungi, biofilm, autofluorescence.

Introduction

The basic principle of RNA targeted *in situ* hybridization is that a DNA-oligomer that is complementary to a region of the rRNA forms a hybrid with the target region inside morphologically intact cells. The hybrids can be detected by light microscopy because the probe is marked with a fluorescent dye. Probes can be designed for phylogenetically relevant regions of the small ribosomal subunit (SSU) and thus a taxon-specific detection of organisms is possible. The basis for the method was originally developed by John *et al.* (1969) who used radioisotopes for labeling. Later Amann *et al.* (1995) modified the method by using fluorescence marked probes for the *in situ* detection of bacteria in natural samples as water and soil (Manz *et al.*, 1993; Hahn *et al.*, 1992). Until now the method is mainly used in bacteriology and numerous protocols and taxon-specific probes for bacteria have been published (Amann *et al.*, 1995, with extended reference list).

Due to the characteristic cellular properties in fungi, such as rigid walls, large vacuoles, accumulation of secondary metabolites and protein binding sites on the SSU rRNA, the method can not be simply transferred from bacteriology to mycology. Thus only few studies exist on the application of the method for fungi. These studies refer to industrially or medically important yeasts, such as species of *Candida*, *Dekkera* and *Saccharomyces* (Kosse *et al.*, 1997; Lischewski *et al.*, 1996). Li *et al.* (1997) used the method for the identification of *Aureobasidium pullulans* (De Bary) G. Arnaud on leaves. Sterflinger *et al.* (1998) were the first to present a protocol of *in situ* PCR followed by DNA targeted *in situ* hybridization in fungal cells.

In situ hybridization is of special importance for studies on black yeasts and meristematic fungi because of the following reasons: (A) Members of this group are often difficult to isolate and are outcompeted by fast growing saprophytes. Thus an analytical tool for *in situ* detection is necessary. (B) It is impossible to distinguish the taxa by morphology on the natural substrate because meristematic growth is an ecotype occurring in several ascomycete orders (Sterflinger *et al.*, 1999) as response to environmental conditions (Sterflinger & Krumbein, 1995). (C) Meristematic fungi and black yeasts are especially found as inhabitants of organic and inorganic surfaces and thus an *in situ* procedure that can be applied on material surfaces is needed.

It was the aim of this study to present a detailed protocol for *in situ* hybridization that can serve as a basis for different fields of applications. We also wanted to find the potentials and the limitations of the method and to give a stimulus to other researchers to establish this valuable method in mycological studies.

Table 1. Probes used for *in situ* hybridization. The probe names were standardized according to Alm *et al.* (1996). The number indicates the 5´ position on the nucleic acid sense strain complementary to the 3´end of an antisense probe.

Probe name	Sequence from 5´ to 3´	Specificity
S-*-Univ-1392-a-A-18 [1]	g(A/T)ATTACCgCggC(g/T)gCTg	Universal (Amann *et al.*, 1995).
S-K-Fungi-0521-a-A-17 [2]	TAAggg(A/g)TTTA(A/g)ATTgT	Kingdom Fungi with exceptions.
S-G-Conio-1361-a-A-20 [2]	CAACCCACAAAAgTgAgTTg	Genus *Coniosporium*.

[1] the target position is indicated according to the 16S rDNA of *Escherichia coli*.
[2] the target position is indicated according to the 18S rDNA of *Neurospora crassa*.

Table 2. Alignment of bases 1361 to 1380 in the 18S rDNA of 16 meristematic fungi and black yeasts with *N. crassa* as leading sequence.

1361 1380	
|----------|	
CGTATTGCTTTGGCAGTACG	*Neurospora crassa*
CAACTCACTTTTGTGGGTTG	*Coniosporium perforans*
CAACTCACTTTTGTGGGTTG	*Coniosporium apollinis*
CAACTCACTTTTGTGGGTTG	*Coniosporium* sp.
AGGTTCACTTAGGTGGGACG	*Exophiala jeanselmei*
AGGTTGACTTTTGTCGGCCG	*Sarcinomyces phaeomurifomis*
GTGCTACGATTTGCTGGTTA	*Saccharomyces cerevisiae*
AGGCTAACTTTGGTTGGTCG	*Alternaria alternata*
CGGCCCGCTTTGGCGGGTCG	*Aureobasidium pullulans*
AGGTTGACTTTTGTCGGCCG	*Exophiala dermatitidis*
AGGTTGACTGTGGTCGGCCG	*Exophiala moniliae*
AGGCTCACTTAGGTGGGCCG	*Exophiala salmonis*
AGGCCCGCTTTGGCGGGTCG	*Hortaea werneckii*
CAACTCACTTGTGTGGGTTG	*Phaeococcomyces catenatus*
CGGCCAGCTTTGGCTGGTCG	*Phaeosclera dematioides*
AGGTTCACTTTTGTCGGCCG	*Sarcinomyces crustaceus*
AGGCCCGCTTTGGCGGGTCG	*Capnobotryella renispora*

Material and methods

CULTIVATION. – All strains are maintained at the Geomicrobiology group of the University Oldenburg. The list of fungi is given with the results in Table 3. Strains were cultured on 2% malt extract agar (MEA). To grow the fungi on material surfaces small pieces (4 × 4 mm) of glass and plastic (polyethylene) were sterilized and put into Erlenmeyer flasks containing 100 ml liquid malt extract medium. The flasks were inoculated with pieces of mycelia and incubated at room temperature until a well developed film of fungal mycelia was observed on the surface of the materials.

AUTOFLUORESCENCE TEST. – 24 Strains including several hyphomycetes, meristematic fungi and black yeasts were grown on 2% malt extract agar (MEA). Pieces of fungal mycelia were mounted in a drop of water and examined with an Zeiss Axioplan equipped for epifluorescence microscopy with a 50 W mercury high pressure bulb and 2 different sets of filters: Filter No. 9 (excitation filter 450-490 nm, dichroic mirror 510 nm) and filter No. 14 (excitation filter 510-560 nm, dichroic mirror 580 nm). The first filter is specific for the detection of the fluorescent dye fluorescein with green emission and the second for rhodamin with red emission. Photos were taken on Kodak Ektachrome P1600 film with an exposure time between 0.25 and 14 seconds.

PROBES. – The three different probes used for the development of the method are shown in Table 1. The first one was a universal probe for bacteria and eukaryotes (Amann *et al.*, 1995).

Secondly a probe with specificity for a wide range of ascomycetes was designed on the basis of the Antwerp databank on small ribosomal subunit of fungi (URL http://rrna.uia.ac.be) and the EMBL databases on small ribosomal subunits of prokaryotes and plants (URL http://www.ebi.ac.uk).

The third probe was developed as genus-specific for the detection of *Coniosporium* based on the sequences of *C. perforans* Sterflinger (CBS 885.95T), *C. apollinis* Sterflinger (CBS 352.97T) and *Coniosporium* sp. (CBS. 665.80) (Sterflinger *et al.*, 1997), sequences of meristematic fungi (Sterflinger *et al.*, 1999) and sequences of black yeasts (Haase *et al.*, 1995). The alignment of the *Coniosporium* target region with *Neurospora crassa* and representatives of black yeasts and

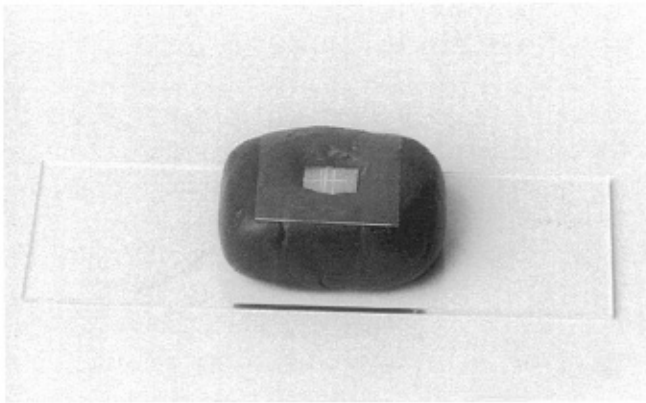

Plate 1. Hybridization chamber for *in situ* hybridization on biofilms on synthetic materials.

meristematic fungi is shown in Table 2. All probes were synthesized marked with rhodamin at the 5´ end by MWG Biotech (Munich, Germany).

The melting temperatures of the probes and their target regions were calculated according to the formula of Leitch *et al.* (1994). The formula is based on the different G/C content of the DNA / RNA. The T_m for the universal probe is 78°C, for the fungal probe 63.4°C and for the *Coniosporium* specific probe 74.2°C. However, the optimum hybridization temperature below the melting temperature was experimentally tested.

Preparation of samples for hybridization. – Fungi grown in 2% malt extract liquid medium were filtered on a Schleicher & Schüll filter (S&S 589), washed with buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) and 0.01 g of the cell material were transferred to Eppendorf tubes. Then 200 µl β-glucanase (8.3 U in 400 µl Tris-buffer; 1 M, pH 6) were added and the mixture was incubated at 55°C for 6 h. The β-glucanase solution was removed with a pipette and the fungal cells were washed three times with PBS buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4 in ddH_2O, pH 7.0). After removing the PBS buffer, 500 µl formaldehyde (4% in PBS buffer) were added and cells were fixed overnight at 4°C. Formaldehyde was eliminated, the cells were washed several times with PBS and stored at -20°C in PBS-ethanol (1:1, v/v).

For hybridization of fungi on synthetic surfaces special chambers were built on object-slides with an inert putty (Terostat, Teroson) (Plate 1). A nut-sized ball of the putty was pressed on the object slides and a dimple of the size of the sample was pressed into the putty. This dimple later serves as the hybridization chamber which can be closed by carefully pressing a large cover slide on it. A small air space was left between the sample and the cover-slide.

In situ hybridization. – For the pure cultures object slides were cleaned with ethanol (70%), coated with a thin layer of gelatin (0.1%) and equipped with frames (25 µl volume, Biozym, Oldendorf, Germany). Five µl of the fixed cell material were pipetted on the area surrounded by the frame and dried at room temperature. The material was dehydrated in ethanol (50%, 80% and absolute, each step for 3 min.). Ten µl of hybridization solution (0.02% SDS, 20 mM Tris buffer, 5 mM EDTA, 0.9 M NaCl, pH 7.4) and 1 µl of probe (50 ng/l) were added. Object slides without probe served as a control for the detection of autofluorescence. Hybridization was carried out in a Perkin Elmer hybridization block for 1.5 hours at 46°C. Alternatively, hybridization can be carried out in a hybridization oven. The object slides then are placed in small plastic chambers (e.g. Greiner tubes) that are equipped with filter paper soaked with hybridization solution to create a humid climate. Evaporation of the hybridization solution must be avoided, to prevent the probes from binding unspecifically to cell material so that they cannot be removed in the consecutive washing steps. Modifications for the *Coniosporium* specific probe are described below.

For hybridization on test materials the fungal probe was used. All steps were carried out in the putty chambers. All temperatures, times and washing steps were the same as for the pure cultures but due to the size of the sample with different amounts of the solutions, 80 µl of hybridization solution and 20 µl of probe (50

Table 3. Autofluorescence of fungi tested.

Strain	Filter set No. 9 (green emission)	Filter set No. 14 (red emission)
Sarcinomyces crustaceus	-	-
Trimmatostroma abietis	-	-
Phaeotheca fissurella	-	-
Phaeosclera dematioides	±	±
Taeniolella faginea	-	-
CBS 884.95, black yeast	+	±
Coniosporium perforans	+	±
Exophiala sp.	-	-
Aureobasidium sp.	+	-
Aureobasidium pullulans	+	+
Phoma sp.	+	±
Penicillium sp.	+	+
Scytalidium sp.	-	-
Paecilomyces sp.	+	+
Penicillium sp.	+	-
Cladosporium sp.	-	-
Cladosporium herbarum	±	-
Gelasinospora sp.	+	+
Penicillium sp.	+	-
Aspergillus sp.	±	-
Alternaria alternata	+	±
Trichoderma harzianum	+	-
Phoma putamicum	+	+
Alternaria sp.	+	+

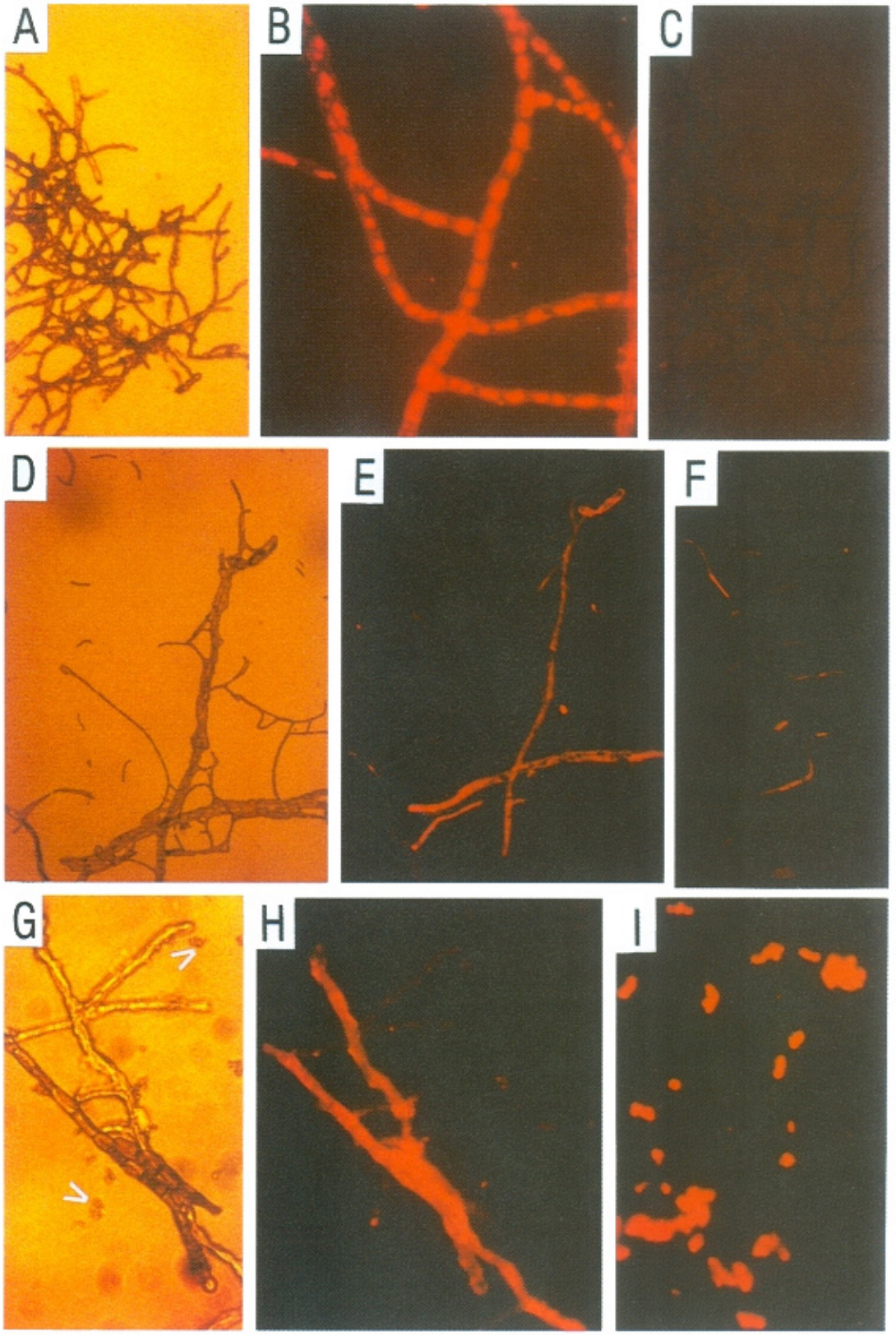

Plate 2. **(A)** Bright-field microscopy of *Coniosporium perforans* (×263). **(B)** The cytoplasm of *C. perforans* is brightly fluorescent after *in situ* hybridization with the universal probe (×608). **(C)** No autofluorescence could be detected in *C. perforans* (×264). **(D)** Bright field microscopy of *C. perforans* and *B. megaterium* (×264). **(E)** After *in situ* hybridization with the fungal specific probe only the fungal hyphae can be detected (×264). **(F)** *In situ* hybridization with *B. megaterium* was carried out as a control (×264). **(G)** Bright field microscopy of *C. perforans* and conidia of *A. pullulans* (exemplary indicated by arrows) (×608). **(H)** *In situ* hybridization of a mixed culture of *A. pullulans* and *C. perforans* (×608). **(I)** Hybridization signal in *A. pullulans* with the fungal probe (×880).

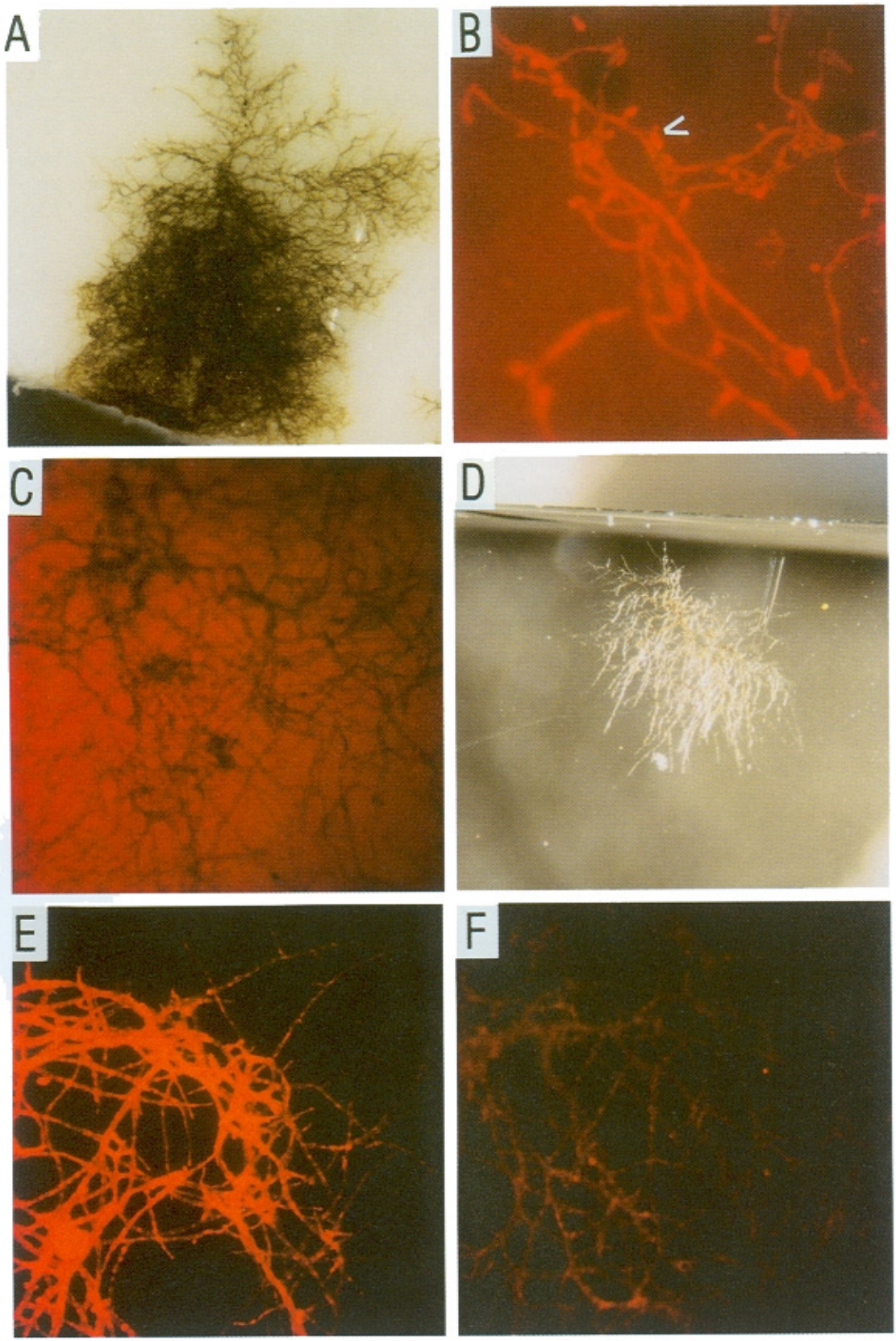

Plate 3. (**A**) *Exophiala* sp. grown on a polyethylene surface (×42). (**B**) *In situ* hybridization of *Exophiala* sp. on polyethylene. Hyphae and budding conidia show a bright red fluorescence that can be distinguished from the background fluorescence of the material. A budding conidium is indicated by an arrow (×608). (**C**) *Exophiala* sp. grown on plastic does not exhibit autofluorescence (×264). (**D**) *Coniosporium perforans* grown on a glass surface (×42). (**E**) The hybridization signal of *C. perforans* on the glass is clearly stronger than the autofluorescence of the fungus shown in (**F**) (×264).

ng/µl) were added and hybridization was carried out in a Perkin Elmer hybridization block. For washing, 150 ml of prewarmed hybridization solution were used.

After hybridization the samples were briefly rinsed with ddH_2O and then washed twice in closed chambers with prewarmed hybridization solution at 48°C for 10 min. The washing temperature was higher than the hybridization temperature to remove unspecific bindings. Samples were again rinsed with ddH_2O, dried at room temperature and observed with a Zeiss Axioplan with filter set 14. To preserve the fluorescence, samples were covered with Vectashield mounting medium (Vecta Laboratories, Burlingame, U.S.A.) and stored in the dark.

MODIFICATIONS OF THE HYBRIDIZATION PROCEDURE FOR THE *Coniosporium*-SPECIFIC PROBE. – The hybridization temperature for the probe was 46°C. Because the probes sequence has a tendency for self-assembly the probe was denatured before use in a waterbath at 80°C for two minutes. In order to optimize the strength of the signal the formamid concentration was varied from 5% to 25% in steps of 5%. Additionally the cell material fixed on the object slides was treated with 50 µl HCl (200 mM), incubated at room temperature for 30 min and rinsed with ddH_2O. The dehydration and hybridization was done as described above. Also a treatment with proteinase K was tested; 25 µl proteinase-K stock solution (10 U/ml proteinase K, 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4) were pipetted on the cell material and fixed on the slides as described above. The slides were covered with cover slips, incubated for 19 h at 37°C and washed with ddH_2O.

Results

AUTOFLUORESCENCE OF FUNGAL STRAINS. – The results of the test are shown in Table 3. Using filter set No. 9 which is specific for the detection of fluorescein-marked probes only 10 isolates showed a weak or no autofluorescence. With the rhodamin-specific filter-set No. 14, 18 of 24, isolates tested exhibited only weak or no autofluorescence. For this reason rhodamin-marked probes were used for the hybridization experiments. For the material experiments fungal strains with weak or no autofluorescence were chosen.

HYBRIDIZATION WITH THE UNIVERSAL PROBE. – The hybridization with the universal probe resulted in brightly red fluorescence for all fungi tested. This shows that the conditions for the hybridization procedure were appropriate. The entry of the probe into the cells was achieved by the glucanase treatment without the loss of morphological structures. Plate 2B shows the results of the hybridization with the universal probe. A bright red fluorescence can be seen in the cytoplasm of *Coniosporium perforans*. The negative control shows that no autofluorescence hampers the detection of the signal (Plate 2C).

DESIGN OF A FUNGAL SPECIFIC PROBE. – By using the EMBL databanks for SSU of fungi, plants and bacteria it was not possible to find a region in the rRNA gene that was only found in fungi but not in algae or bacteria. We managed to design a probe that is suitable for a wide range of ascomycetes and that does not fit to bacteria. As an example, Plate 2D shows a mixture of *Coniosporium perforans* and *Bacillus megaterium*. It can be seen in Plate 2E that a signal with the fungus specific probe occurs in the fungal cells but not in the bacterial cells. As a control a hybridization in *B. megaterium* was carried out with a 16S rRNA targeted probe and this did only result in a signal in the bacterial cells (Plate 2F).

***Coniosporium*-SPECIFIC PROBE.** – By using the probe that was specifically designed for the genus *Coniosporium* a signal could only be detected in strains of this genus. Using the same hybridization parameters as for the fungal probe the signal of the *Coniosporium* probe was weak. Neither a treatment with HCl nor a digestion of proteins possibly masking the ribosomal RNA helped to get a more intensive signal. The formamide concentration was the decisive factor for this reaction. Its optimum was at 15%, below and above this concentration the hybridization resulted in a weaker signal. With this formamide concentration a signal can be achieved in all strains of *Coniosporium* but not in the other meristematic fungi tested. As an example, Plate 2H shows the *in situ* hybridization with a mixture of *Coniosporium perforans* and conidial masses of *Aureobasidium pullulans*. The conidia of *A. pullulans* can only be detected by their weak autofluorescence. A real hybridization signal in *Aureobasidium* was carried out with the fungal probe as a control and gave a strong signal (Plate 2I).

***In situ* HYBRIDIZATION ON TEST MATERIALS.** – Fungal hyphae on material surfaces can well be detected by the help of the *in situ* hybridization method. In Plate 3A a colony of *Exophiala* sp. grown on a polyethylene surface is shown. The hybridization resulted in a bright fluorescence of both hyphae and conidia, although the background fluorescence is relatively strong. Autofluorescence of this strain was not observed (Plate 3C). An example of *C. perforans* grown on glass is given in Plate 3D. The hybridization signal (Plate 3E) differs significantly from the autofluorescence shown in Plate 3F. The signal of the fungal cells can also be discriminated from the background fluorescence of the polyethylene.

The hybridization was possible on all non-porous materials that did not exhibit a strong autofluorescence as polyethylene, aluminum and glass. In strongly porous materials the method failed because the hybridization solution leaked into the pores and could not be washed off, thus resulting in a strong fluorescent background. Attempts to carry out a hybridization on marble embedded in LR White (according to Leitch *et al.*, 1994) failed. In a few cases a strong autofluorescence occurred in fungi adhered to materials, although the cultures did not exhibit autofluorescence when grown on agar. In this case a detection of the probe signal was impossible.

Discussion

The protocol presented here can be used as a basis for the specific detection of fungi by *in situ* hybridization. Permeabilization of the cell wall for the probe can be achieved by glucanase treatment. It is not necessary to use the more specific but also far more expensive chitinase for the digestion of the cell walls. The treatment is sufficient for a wide range of meristematic fungi with thick melanized walls but it is also sensitive enough to preserve the morphology of thin-walled hyphomycetes. In Plate 3B even the morphology of the thin-walled budding conidia of *Exophiala* sp. and their daughter cells are seen to be preserved. Fixation with 4% formaldehyde worked for all fungi tested although paraformaldehyde or methanol-acetic acid might be alternatively used for the fixation of black yeast cells (Li *et al.*, 1997).

The optimal hybridization temperature and the concentration of the formamide must be experimentally tested for different probes. In case the probe signal is too low, as it was the case for the *Coniosporium* specific probe, a higher or lower formamide concentration can help to increase the signal if intramolecular interactions are the reason for the low signal. Formamide reduces the melting temperature between DNA strands and thus the denaturation of the DNA-DNA and DNA-RNA duplexes can be achieved at lower temperatures not leading to deterioration of morphology. A treatment with HCl and proteinase-K might help to increase the target accessibility in case the rRNA is masked by proteins (Rolighed & Lindeberg, 1996). Because the accessibility of different sites of the rRNA molecule is different, there is no basic rule for the right formamid concentration. It has to be experimentally tested for every new probe.

On non-porous materials the hybridization can be carried out in the chambers described in this study. For all materials tested, the fluorescence of the fungi could clearly be distinguished from the background of the material. This is different for porous material in which the probe might penetrate and cause a strong fluorescent signal. As described in clinical samples and tissue (Ge *et al.*, 1997) the material was embedded in paraffin and in LR White but it was not possible to get satisfactory results (Hain, 1998).

A problem of the method is still the autofluorescence of a wide range of fungi. The strongly melanized fungi tested in this study exhibited none or little autofluorescence. Thus the autofluorescence does not seem to be a problem caused by the melanins in the cell walls but rather of secondary metabolites as e.g. antibiotics, isoprenoids, carotenes and other substances with aromatic rings or double bindings that are found in hyphomycetes such as *Penicillium*, *Aspergillus* or *Trichoderma* (Griffin, 1981). Some fungi only exhibit autofluorescence when attached to a material surface. This indicates that extracellular polymeric substances excreted for adhesion are the fluorescent factor. This problem can possibly be solved by marking the probes with non-fluorescent labels as e.g. digoxigenin or biotin. The applicability for the detection of fungi in wood and in epizoic marine biofilms is currently tested in the Geomicrobiology group. In general it can be stated that it is necessary to the check samples for autofluorescence prior to hybridization with fuorescent probes.

Still the most important factor hampering the broad application of the method is the lack of sequencing data in the databanks. In contrast to bacteriology, where thousands of sequences of the small and the large ribosomal subunit are available for the design of taxon specific probes, only several hundreds of fungal species were sequenced to date. Thus for the application of the method described here more sequencing work will have to be done and has to be made available in the databanks. Some fungus-specific primers and group-specific oligonucleotides were published by Kappe *et al.* (1996).

The potential applications of the method are numerous. It can help to get a deeper understanding of fungal ecology because it allows a semi-quantitative detection of fungi in natural samples and to visualize their spatial distribution; it helps to discriminate fungi with similar morphology. The method can be valuable in different environments of mixed fungal communities as e.g. soil, marine sediments, biofilms on materials, plants or animals, plant pathogens or lichen parasites.

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