INTERNATL MICROBIOL (2000) 3:183–186

RESEARCH ARTICLE

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Colorimetric in situ hybridization (CISH) with digoxigenin-labeled oligonucleotide probes in autofluorescent hyphomycetes

Received 12 May 2000
Accepted 6 July 2000

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Summary We used digoxigenin-labeled probes for in situ hybridization of hyphomycetes to replace the commonly used fluorescent proof of probe binding by a colorimetric reaction. The resulting blue-purple, intracellular precipitate could be easily detected by light microscopy, and thus presented a promising method to overcome autofluorescence of fungal material and substratum. Optimal cell fixation and permeabilization procedures, as well as hybridization conditions were developed on the example of two different hyphomycetes: *Phialophora* sp. and Cartapip™, a colorless mutant of *Ophiostoma piliferum* (Agra Sol).

Key words *Phialophora* · Digoxigenin-labeled probes · Hyphomycetes · Autofluorescence · Colorimetric in situ hybridization

Introduction

In situ hybridization was originally developed in bacteriology for taxon specific detection of procaryotes without cultivation [1]. Therefore, fluorescence-labeled, rRNA-targeted probes were used because of their easy handling and detection. Fluorescence in situ hybridization (FISH) was not only applied to study certain groups of bacteria [8], but also to detect them in their natural environment [7]. Only recently was the method modified and adapted to the requirements of a certain group of hyphomycetes [12], and has now been used to detect *Aureobasidium pullulans* on leaf surfaces [5, 10] and yeasts in yogurt [4]. Pathogenic *Candida* species could also be detected in a human endothelial cell line [6]. Furthermore, in situ PCR followed by FISH was used to detect slow growing fungi with low metabolic activity with 18S rDNA-targeted probes [11]. When using FISH for the detection of epi-/endophytic and epi-/endozoic hyphomycetes, the detection is often hampered by the strong autofluorescence either of the materials or, particularly, of the associated fungi. To overcome this problem, digoxigenin (DIG)-labeled probes can be used [3, 13]. Probe binding can be detected colorimetrically by antibodies specific for digoxigenin, which are coupled with an enzyme (alkaline phosphatase). After adding the enzyme substratum (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate), an intracellular blue-purple precipitate occurs, easily to detect by light microscopy. Thus, colorimetric in situ hybridization (CISH) is a promising method to overcome autofluorescence of fungal material and substratum. In this study we report the results of CISH on the example of two different hyphomycetes—*Phialophora* sp. and Cartapip™ (a colorless mutant of *Ophiostoma piliferum*, Agra Sol)—and a first protocol for the application to hyphomycetes.

Materials and methods

Growth conditions, cell permeabilization and fixation Fungal strains of Cartapip™ (Agra Sol) and *Phialophora* sp. were cultivated in liquid malt extract media (2%) for 4–10 days. Cultures were harvested by filtering, and washed with PBS-buffer (1 M, pH 7). Approximately 0.05 g of fungal material were incubated with 250 µl of β-glucanase (10 U/ml in 1 M Tris/HCl buffer, pH 6) for 2.5–6 hours at 55°C and washed three times with PBS-buffer (s. a.). To preserve cell morphology, cells were fixed in 4% paraformaldehyde solution (in PBS-buffer) at 4°C overnight. Subsequently, the cells were washed again in PBS-buffer (s. a.) and stored in PBS/ethanol (1:1 v/v) at –20°C [12].

Oligonucleotide probes In this study the following probes were used: (i) universal probe (5´-GWA TTA CCG CGG CKG CTG-3´)[2]; (ii) non-universal probe (5´-CAG CAG CCG CGG

TAA TTC-3´), which is complementary to the universal probe and served as negative control for non specific-binding [5]. Both probes were labeled at the 5´-end with digoxigenin (DIG).

In situ hybridization using the digoxigenin-labeled universal and non-universal probes Colorimetric in situ hybridization was principally carried out according to the protocol of Zarda et al. [13], developed for bacteria, but was adapted to the properties of *Phialophora* sp. and Cartapip™.

Fixed cell material was dropped on cleaned, chambered glass slides (Carl Roth GmbH & Co., Karlsruhe), air-dried, dehydrated in 50%, 80% and absolute ethanol and dried at room temperature. Samples of 8 µl hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 0.01% SDS, 10–30% formamide, pH 8) and 1 µl of the universal/non-universal probe (50 ng/µl) were pipetted to each well of the prepared slides, and incubated in an equilibrated humid chamber at 46°C for 1.5 hours. The universal probe served as a positive and the non-universal probe as a negative control. Additional negative control samples were hybridized without probes. After hybridization the slides were rinsed with distilled water. Optionally, the slides were washed stringently at 48°C for 20 min with hybridization buffer, rinsed with distilled water and air-dried.

For the detection of DIG-labeled hybrids, an antibody solution was pipetted to the cells. Therefore, specific anti-DIG-antibodies (Fab-fragment; Boehringer Mannheim) coupled with alkaline phosphatase (AP) were used. Anti-DIG-antibodies were diluted in a buffer containing 150 mM NaCl, 100 mM Tris/HCl (pH 7.5) and 0.5% blocking reagent (Boehringer Mannheim) to a final concentration of 2.5–5 U/ml. Aliquots of 10 µl of this solution were pipetted to each well with the hybridized samples and incubated in an equilibrated humid chamber for 1 hour at 27°C. Additional negative control samples (hybridized without probes) were treated with 10 µl of anti-DIG-AP dilution and 10 µl of the antibody buffer, respectively. Subsequently, the slides were washed in a washbuffer (150 mM NaCl, 100 mM Tris/HCl, 0.01 SDS, pH 7.4) for 10 min at 29°C and air-dried. For the detection of the enzyme-conjugated anti-DIG-antibodies, a substrate stock solution of NBT (nitroblue-tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolylphosphate, toluidine salt) was purchased from Boehringer Mannheim. This stock solution was diluted in 100 mM Tris/HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$, according to the protocol of Boehringer. An aliquot of this substrate solution (30 µl) was pipetted to each well including the negative control samples. The slides were incubated inside a humid chamber at room temperature overnight. The insoluble, blue-purple product was visualized by light microscopy using bright field. Color photographs were taken on Kodachrome EPY 64T.

Results and Discussion

When using fluorescent in situ hybridization for the detection of Cartapip™ and *Phialophora* sp. on natural samples, the detection is hampered by the strong autofluorescence of the substratum (wood and bryozoan) and/or the fungi from the biofilm growing on these materials. To overcome this problem of autofluorescence, colorimetric in situ hybridization was used for the detection of Cartapip™ and *Phialophora* sp., and the first results are presented. After hybridization with the DIG-labeled universal probe, antibody binding and addition of the substrate solution to the cells of Cartapip™ and *Phialophora* sp., we observed a blue-purple precipitate in the cells (Fig. 1A and 2A). One key parameter for successful hybridization is the permeabilization of cell walls, in order to admit penetration of the large molecules (antibody-enzyme complex) of the indirect probe detection system [10, 11, 12]. On the other hand, the stringency of probe binding could be increased using different formamide concentrations (10% for Cartapip™ and 30% for *Phialophora* sp.). As a result, unspecific binding of the non-universal probe could be minimized for both fungal strains (Fig. 1B and 2B). The consequence of the optional washing step after hybridization also was higher probe binding stringency. However, the additional washing step can cause considerable loss of cell mass on the slide. And in the case of Cartapip™, it could not increase the stringency more than formamide in the hybridization buffer did. Altogether, in any case only the best results are presented. To use the method in mixed assemblages, a mean formamide concentration (i.e. 20%) could be used.

To test the specificity of the anti-DIG-antibody, we treated the second negative control sample only with the specific antibody and its substrate solution. The results for Cartapip™ showed only weak (Fig. 1C) and for *Phialophora* sp. stronger unspecific binding of the antibody (Fig. 2C). This might be due to the higher antibody concentration used for the treatment of *Phialophora* cells, 5 U/ml in contrast to 2.5 U/ml for Cartapip™. Higher antibody concentrations were used with *Phialophora*, because the fungus differs in pigmentation from Cartapip™. (The latter is colorless, *Phialophora* is pigmented slightly brown). Pigmentation changes the effect of permeabilization conditions, because it results in stronger cell walls. And furthermore, probe detection needs a stronger signal to overcome the pigmentation. Thus, the concentration of the antibody solution appeared to be crucial, and we had to optimize it carefully to achieve maximum probe detection and minimum unspecific binding. The negative control samples treated only with substrate solution showed no blue-purple precipitate in the cells. Thus, unspecific coloring due to the activity of alkaline phosphatase within the fungal cells could be excluded (Fig. 1D and 2D). Table 1 gives an overview of significant variables of the protocol, which possibly have to be adapted for the application to other hyphomycetes, and their effect on hybridization results.

This method is a promising alternative to FISH. High probe detection rates and minimal unspecific signal could be achieved after careful optimization of hybridization and probe-detection parameters. Yet, the same system could only be applied to the

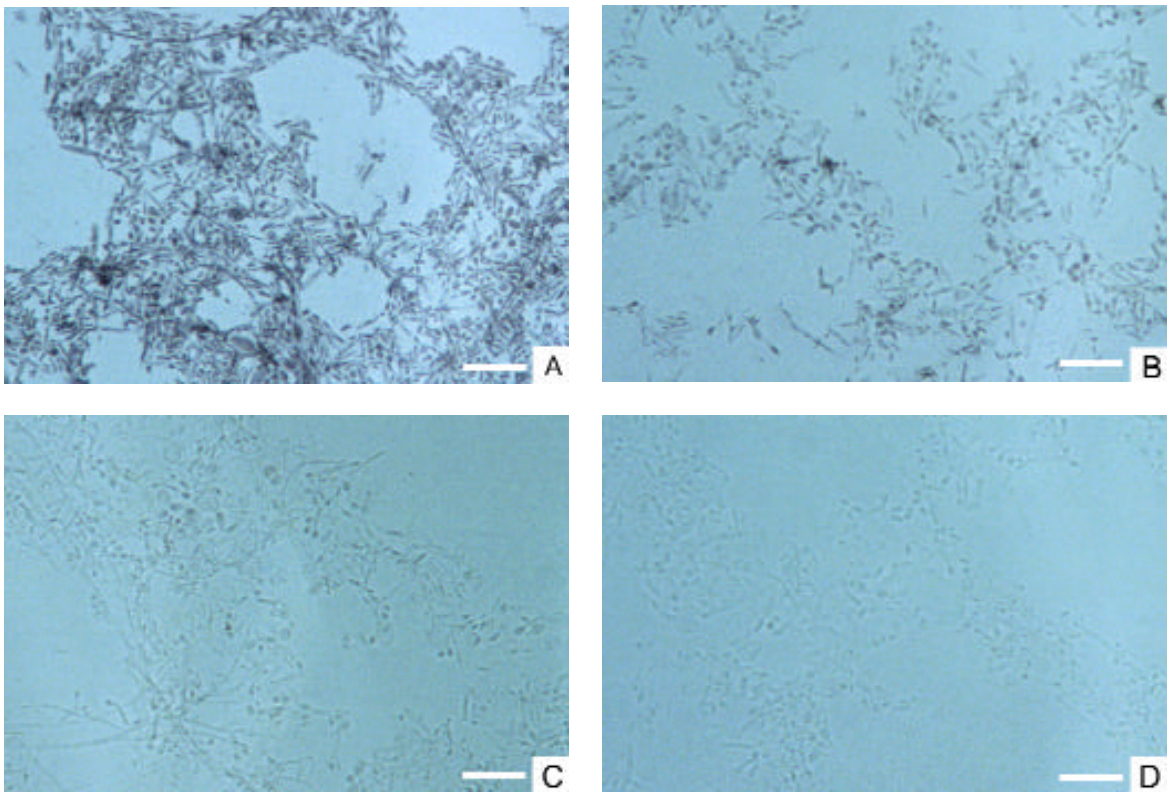

Fig. 1
Colorimetric in situ hybridization of Cartapip™ (bar = 90 µm). (A) Positive control sample with the universal probe. (B) Negative control sample with the non-universal probe. (C) Negative control sample for unspecific binding of the antibody (treated with anti-DIG-antibody and substrate solution). (D) Negative control sample for intracellular alkaline phosphatase activity (treated with substrate solution)

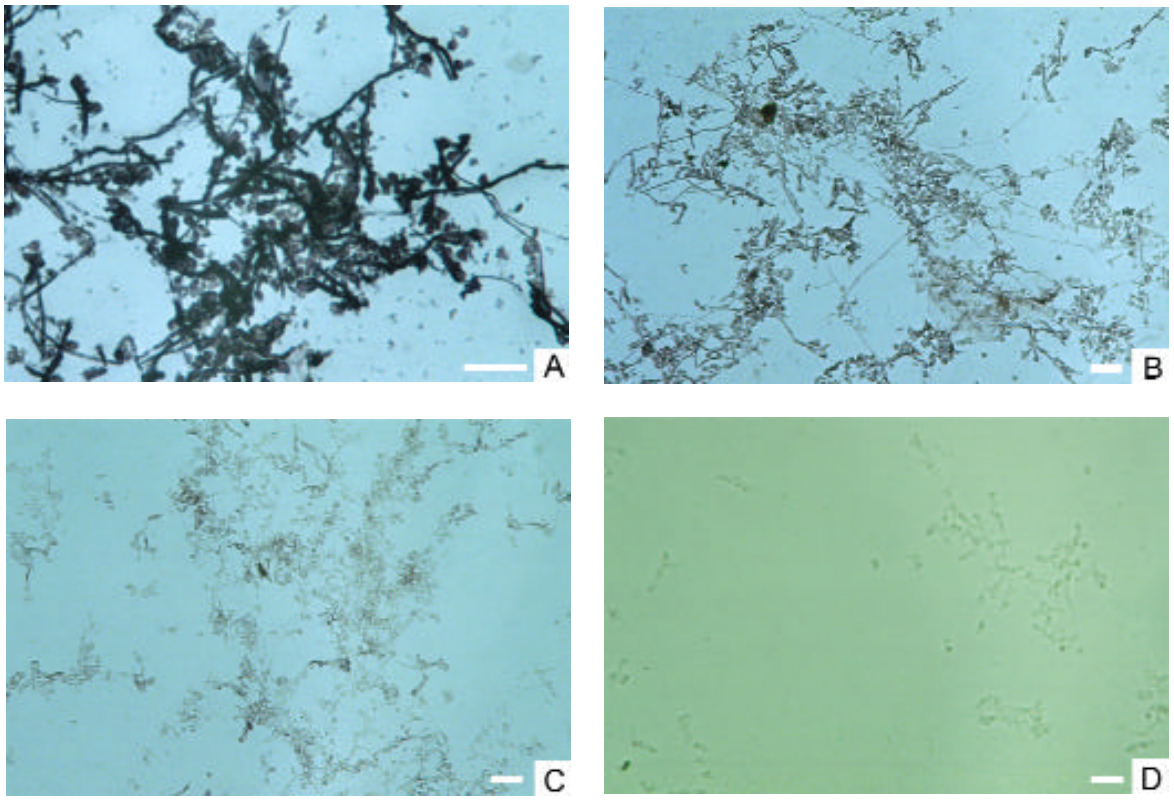

Fig. 2
Colorimetric in situ hybridization of *Phialophora* sp. (bar = 90 µm). (A–D) same as in Fig. 1

Table 1 Variables which have to be adapted and their effect on hybridization results

Variable	Effect on hybridization result
β-Glucanase treatment (5–15 U/ml for 2–6 h)	Optimizing permeabilization of cell walls
Amount of probe (50–100 ng)	Maximizing hybridization Minimizing unspecific binding
Formamide concentration (5–35%)	Optimizing stringency of probe binding
Additional washing step after hybridization	Optimizing stringency of probe binding (Eventually loss of cell mass)
Anti-DIG-AP concentration (1.5–5 U/ml)	Maximizing probe detection Minimizing unspecific binding of the antibody
Blocking reagent (0.5–1%)	Minimizing unspecific binding of the antibody

in situ detection of the actinomycete *Frankia* [14]. Yet, a similar strategy to deal with autofluorescence problems was described for in situ hybridization of cyanobacteria using directly enzyme-labeled (horseradish peroxidase) probes [9]. As direct enzyme-labeling leads to more difficult handling of probes, and detection rates using the antibody-technique as described were very satisfactory, this method was favored for the application to hyphomycetes. Note, however, that it is not possible to present a general protocol of colorimetric in situ hybridization that is applicable on all hyphomycetes. For the detection of different fungi the variables of the protocol probably have to be adapted to the requirements of these fungi as it is presented in this study.

Acknowledgments This work was supported by the "Studienstiftung des deutschen Volkes" and the VW-Stiftung, grant No. 25D.-76 251-10-8/97/4 (ZN 283).

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