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Whole-Cell Hybridization of *Frankia* Strains with Fluorescence- or Digoxigenin-Labeled, 16S rRNA-Targeted Oligonucleotide Probes

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Whole-cell hybridization with non-radioactively labeled oligonucleotide probes was used to detect and identify *Frankia* strains in pure cultures and in nodules. Digoxigenin-labeled probes, which were detected with antibody-alkaline phosphatase conjugates, were more suitable for in situ detection of *Frankia* strains than fluorescent probes since the sensitivity of the former was higher and problems arising from the autofluorescence of cells and plant material were avoided. Successful detection of *Frankia* strains in paraformaldehyde-fixed cell material with digoxigenin-labeled oligonucleotide probes depended on pretreatments to permeabilize the cells. Specific hybridization signals on vesicles were obtained after lysozyme pretreatment (1 mg ml^{-1} for 30 min at 20°C). Reliable penetration of the antibody-enzyme conjugate into hyphae required additional washing with the detergent Nonidet P-40 (0.1%) and toluene (1% in ethanol) after lysozyme treatment. Identification of *Frankia* vesicles in nodule homogenates was possible only after the removal of the polysaccharide capsule surrounding the vesicles. Incubation with H_2O_2 (15% in water for 1 h at room temperature) before lysozyme and detergent treatments was found to facilitate specific hybridization. No filaments or spores could be detected in nodule homogenates. This technique should be a powerful tool in the identification of *Frankia* isolates, in the characterization of as-yet-uncultured nodule populations, and in the confirmation of the origin of unusual *Frankia* isolates.

The members of the actinomycete genus *Frankia* are slowly growing microorganisms which form root nodules in symbioses with woody plants (10). For a long time, systematic and ecological studies of *Frankia* strains have been hampered by the lack of reliable morphological, physiological, and cytochemical markers. Problems in isolation and identification of this organism have been attributed to the long generation times, the requirement of special isolation factors (34), the lack of specific nutritional requirements of the pure cultures obtained so far (2), and the great variability of the isolates combined in the genus *Frankia* (1, 16, 17, 20, 29, 30). Today, identification of isolates and delineation of the genus *Frankia* can be done reliably on the basis of fatty acid patterns (26, 30, 36) or rRNA sequences (20–22, 24, 29, 31). By using these methods, many atypical isolates, which often lack the ability to fix nitrogen or to form root nodules on their original host plants, have been identified as *Frankia* sp. (20, 29, 30). The origin of these non-nodule-forming isolates (that is, whether they belong to the nodule population or are surface contaminants) often remains questionable.

The origin of an isolate can be confirmed by a combination of rRNA or ribosomal DNA sequence retrieval (often polymerase chain reaction assisted) and in situ hybridization of fixed samples (5, 13, 14, 37). The labor-intensive methods based on probes labeled with radioisotopes (18) have stimulated the search for alternative labeling strategies. rRNA-targeted oligonucleotides that are labeled with nonradioactive reporter molecules like biotin or digoxigenin (14, 40), enzymes (7), or fluorescent dyes (3–6, 11–13, 19) have found wide application in the detection and identification of bacteria. Nonradioactive oligonucleotide probes, however, still present some problems. Because of their low level of sensitivity, fluorescent probes can be used only to detect cells with relatively high rRNA contents (19). Moreover, the use of these probes requires environments exhibiting low levels of autofluorescence. Digoxigenin-labeled probes and subsequent detection of the digoxigenin by alkaline phosphatase-conjugated anti-DIG Fab fragments overcome this problem. However, the cells must be pretreated to increase their permeability, and, up to now, the use of this technique seems to have been restricted to gram-negative bacteria (40).

The feasibility of enzymatic digoxigenin-based detection of target sequences in plant material has been demonstrated previously (9, 38). There have been no problems with background interference when preparations from in situ hybridization experiments performed with digoxigenin-labeled probes have been examined by bright-field microscopy. In situ hybridization on vesicles and filaments of *Frankia* cells in nodules, however, is influenced by additional capsular material surrounding the cells in nodules. The polysaccharide (25) of the capsular material is highly resistant to pectinase, detergent, and proteinase K treatments (8). The permeabilization protocols developed so far probably are not directly transferable to vesicles developed in nodules.

The aim of this study was to examine the applicability of nonradioactive labels to in situ detection and identification of the slowly growing actinomycete genus *Frankia*. Our investigations focused on detection with 16S rRNA-targeted, fluorescence- or digoxigenin-labeled oligonucleotide probes used on cells grown in pure cultures and in nodule homogenates. A critical issue was the permeability of the cell walls and membranes of the fixed organisms, which is essential for the penetration of probes and antibody-enzyme conjugates.

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MATERIALS AND METHODS

Bacterial strains and nodules. Nitrogen-fixing *Frankia* sp. strain Ag45/Mut15 and non-nitrogen-fixing strain AgB1.9 (23) were grown in P+N medium (28) containing propionic acid and ammonium chloride as C and N sources, respectively. Strain Ag45/Mut15 was also grown in the absence of ammonium chloride (P−N medium). *Lactococcus lactis* subsp. *cremoris* ATCC 19257, which was grown on Luria-Bertani medium, was used to evaluate probe selectivity, as were pure cultures of several gram-positive and gram-negative bacteria (19).

Nodules of the spore(+) type were collected from *Alnus glutinosa* on the banks of the River Limmat in Dietikon, Switzerland, in early July.

Pretreatment and fixation of cells. After 1 week of incubation at 30°C, pure cultures of *Frankia* sp. were harvested by centrifugation, washed in phosphate-buffered saline (PBS) (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.2 in water), and fixed at 4°C for 3 to 16 h by the addition of 3 volumes of fixation buffer (4% paraformaldehyde in PBS [pH 7.2]). Nodules (approximately 10 mg [fresh weight]) were fixed in 1 ml of fixation buffer. Cells and nodules were washed in PBS and stored in 1 ml of 50% ethanol in PBS at −20°C until they were used (3).

Before the cells were applied to the slides, they were sonicated mildly to disrupt clumps. Fixed nodules were ground in a mortar and used as nodule homogenates. Samples (1 μl) of cells and nodule homogenates were spotted onto gelatin-coated slides [0.1% gelatin, 0.01% $KCr(SO_4)_2$] and allowed to air dry.

After dehydration in 50, 80, and 96% ethanol (3 min each), the preparations were incubated with lysozyme (1 mg ml^{-1} in 100 mM Tris [pH 7.5]–5 mM EDTA) at 20°C for 0.5 h, washed in 100 mM Tris (pH 7.5)–5 mM EDTA supplemented with 0.1% Nonidet P-40 (Sigma, Basel, Switzerland) for 10 min at room temperature, and dehydrated as described above, except that a toluene treatment (1% toluene in 96% ethanol) was included before the last ethanol dehydration step. Each of the pretreatment steps was also tested as a single step. In addition, proteinase K treatments alone (10 to 100 ng of proteinase K ml^{-1}, with or without 0.1% sodium dodecyl sulfate [SDS]) and before and after lysozyme treatment were tried.

Before the nodule homogenates were subjected to the treatments described above for pure cultures, they were treated with peracetic acid (acetic anhydride–30% H_2O_2 [1:1, vol/vol]) (15) or H_2O_2 (15% in water) for 1 to 4 h at room temperature. In addition, before or after incubations with peracetic acid or H_2O_2, preparations were treated with cellulase (20 mg ml^{-1}; Fluka, Buchs, Switzerland), pectinase (20 mg ml^{-1}; Fluka), or mixtures of the two compounds (1:1) at room temperature for 1, 2, or 4 h.

Probes. The following four oligonucleotide probes were synthesized with a primary amino group at the 5′ end (Aminolink 2; Applied Biosystems, Foster City, Calif.): probe EFP, complementary to the 16S rRNAs (positions 1020 to 1042 in the *Escherichia coli* numbering system) of nitrogen-fixing strains belonging to the *Alnus* compatibility group; probe IFP, complementary to the 16S rRNAs (positions 1020 to 1042) of non-nitrogen-fixing strains (20); bacterial probe Eub338 (4); and eukaryotic probe Euk516 (3). The fluorescent dye tetramethylrhodamine isothiocyanate (TRITC) (Research Organics, Cleveland, Ohio) was covalently bound to the amino group, and the dye-oligonucleotide conjugate (1:1) was purified from unreacted components and stored at −20°C in double-distilled water at a concentration of 50 ng μl^{-1} (3).

Digoxigenin-ddUTP (Boehringer, Rotkreuz, Switzerland) was used to label the four probes at their 3′ ends with terminal transferase in accordance with the manufacturer's instructions.

The DNA-specific dye 4′,6-diamidino-2-phenylindole (Sigma) was stored in a 1-mg ml^{-1} solution at −20°C. A 1-μg ml^{-1} dilution was stored at 4°C and used to stain bacterial cells nonspecifically (33).

Whole-cell hybridization. The preparations were hybridized in 7 μl of hybridization buffer (0.9 M NaCl, 0.01% SDS, 5 mM EDTA, 10 mM Tris [pH 7.2]) containing 1 μl (50 ng) of fluorescent probe at 42°C for 1 h. After hybridization, the slides were washed in hybridization buffer for 20 min at 45°C, rinsed with distilled water, and air dried. Next, 10 μl of a 1-μg ml^{-1} 4′,6-diamidino-2-phenylindole solution was added to each sample, and the preparation was incubated for 5 min at room temperature, rinsed with distilled water, and air dried. The preparations were examined with a microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence detection with a high-pressure mercury bulb and filter sets for 400- and 580-nm light (filter sets 02 and 15).

Hybridizations with digoxigenin-labeled probes were performed under the conditions described above for fluorescent probes, except that a different hybridization buffer (25 mM maleic acid [pH 7.5], 100 mM Tris [pH 7.4], 300 mM $MgCl_2$, 0.01% SDS, 0.5% blocking reagent [Boehringer]) was used. After an additional washing step in 100 mM Tris-HCl (pH 7.5) for 5 min at room temperature, the slides were incubated with 10-μl portions of alkaline phosphatase-conjugated anti-DIG Fab fragments (Boehringer) diluted 1:30 in 150 mM NaCl–100 mM maleic acid–(pH 7.5) 0.5% blocking reagent (Boehringer) at 27°C for 1 h. Then the slides were washed, first in 100 mM Tris-HCl–150 mM NaCl (pH 7.5) and then in 100 mM Tris-HCl–100 mM NaCl–50 mM $MgCl_2$ (pH 9.5) for 5 min at room temperature. Alkaline phosphatase activity was detected by the formation of a water-insoluble precipitate after incubation in 50 μl of substrate solution (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris-HCl–100 mM NaCl–50 mM $MgCl_2$ [pH 9.5]) in accordance with the manufacturer's instructions at room temperature for 3 to 16 h (40). Afterward, the slides were rinsed with distilled water, washed in 10 mM Tris-HCl–1 mM EDTA (pH 8.0) for 5 min, and air dried. The preparations were examined with a Zeiss microscope.

RESULTS AND DISCUSSION

Initial observations. The use of fluorescence-labeled oligonucleotides to detect 16S rRNA sequences in cells from pure cultures of *Frankia* sp. strains Ag45/Mut15 and AgB1.9 resulted in quite weak signals. Neither *Frankia*-specific probes EFP and IFP nor universal eubacterial probe Eub338 strongly hybridized to *Frankia* cells. However, probe Eub338 showed intense hybridization to organisms other than *Frankia* sp. in the same preparation. Only occasionally were signals on fragments of *Frankia* filaments obtained that differed in intensity from the signals on unhybridized controls. However, even these signals could not reliably be attributed to hybridization with the probes because the *Frankia* cells exhibited considerable autofluorescence. Autofluorescence of vesicles and small sporangia was much more pronounced than autofluorescence of the filamentous mass. *Frankia* sp. is a slowly growing organism with generation times of 2 to 3 days. It is unclear whether the low signal

TABLE 1. Influence of pretreatment of *Frankia* vesicles and hyphae on detection by in situ hybridization with fluorescence- or digoxigenin-labeled, 16S rRNA-targeted oligonucleotide probes

Pretreatment(s)	Specific signal obtained with[a]:			
	TRITC-labeled probes		Digoxigenin-labeled probes	
	Vesicles	Hyphae	Vesicles	Hyphae
Lysozyme[b]	+[c]	+	+	–
Nonidet P-40[d]	–[c]	–[c]	–[e]	–[e]
Toluene[f]	–[c]	–[c]	–[e]	–[e]
Lysozyme + Nonidet P-40	+[c]	+	+	+
Lysozyme + toluene	+[c]	+	+	+
Lysozyme + Nonidet P-40 + toluene	+	+	+	+

[a] +, specific signal obtained; –, no specific signal obtained.
[b] Treatment with 1 mg of lysozyme per ml in 100 mM Tris–5 mM EDTA (pH 7.5) for 30 min at room temperature.
[c] Cells exhibited significant autofluorescence.
[d] Treatment with 0.1% Nonidet P-40 in 100 mM Tris–5 mM EDTA (pH 7.5) for 10 min at room temperature.
[e] The level of nonspecific binding of the antibody-enzyme conjugate and the probe in the cells was quite high.
[f] Treatment with 1% toluene in 96% ethanol for 3 min at room temperature.

intensity obtained after in situ hybridization resulted from a low number of target sequences per cell or from low permeability of the gram-positive cells. 4′,6-Diamidino-2-phenylindole staining resulted in very clear pictures of filaments and vesicles, indicating that the cells were penetrated by small molecules (data not shown).

Fluorescent probing of pure cultures of *Frankia* sp. Treatments to increase the permeability of the cells included enzymatic pretreatments (lysozyme, proteinase K) (40), as well as treatments with detergents and solvents (Nonidet P-40, toluene) (39) (Table 1). After pretreatment with lysozyme alone, specific hybridization of TRITC-labeled probes EFP and IFP to the nitrogen-fixing *Frankia* strain and the non-nitrogen-fixing *Frankia* strain, respectively, was obtained (Fig. 1). The fluorescent signal, however, was still quite weak and did not increase after additional treatments (that is, after proteinase K treatment or washing with Nonidet P-40 or toluene).

Fluorescence of vesicles was often much more pronounced than fluorescence of filaments (Fig. 1A). Fluorescent signals of strain Ag45/Mut15 vesicles were obtained after in situ hybridization with probe IFP, which was specific for strain AgB1.9 (Fig. 1B). Fluorescence of vesicles was,

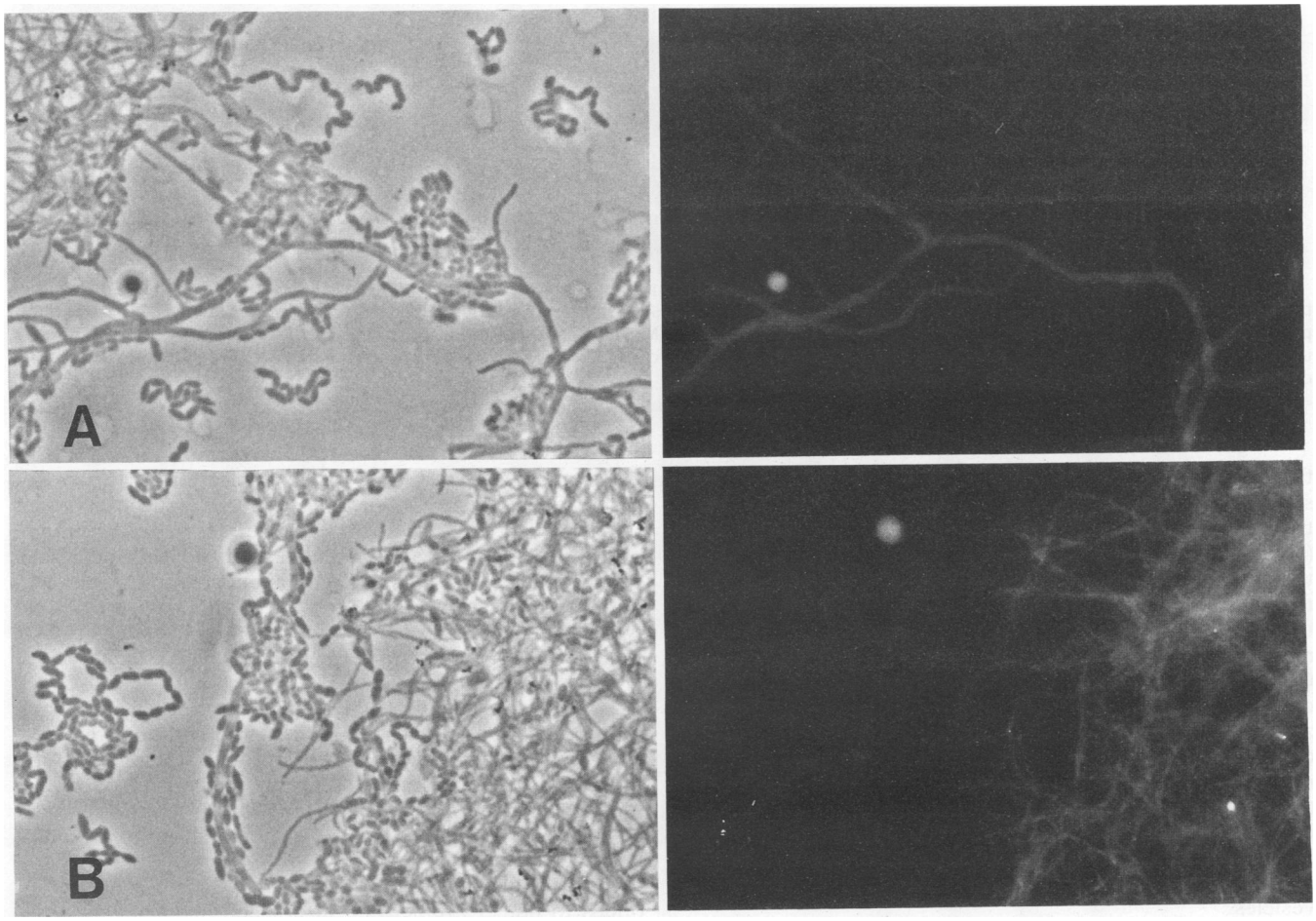

FIG. 1. Specific detection of *Frankia* sp. strains Ag45/Mut15 (A) and AgB1.9 (B) after lysozyme treatment and subsequent in situ hybridization with TRITC-labeled oligonucleotide probes EFP (A, right panel) and IFP (B, right panel), respectively. The intense signals of vesicles obtained with both probes were mainly due to autofluorescence and nonspecific binding of the probes. The corresponding phase-contrast photographs (left panels) show the presence of *Frankia* sp. strains Ag45/Mut15 (large hyphae, vesicles) and AgB1.9 (fine hyphae) and *L. lactis* subsp. *cremoris* (rods).

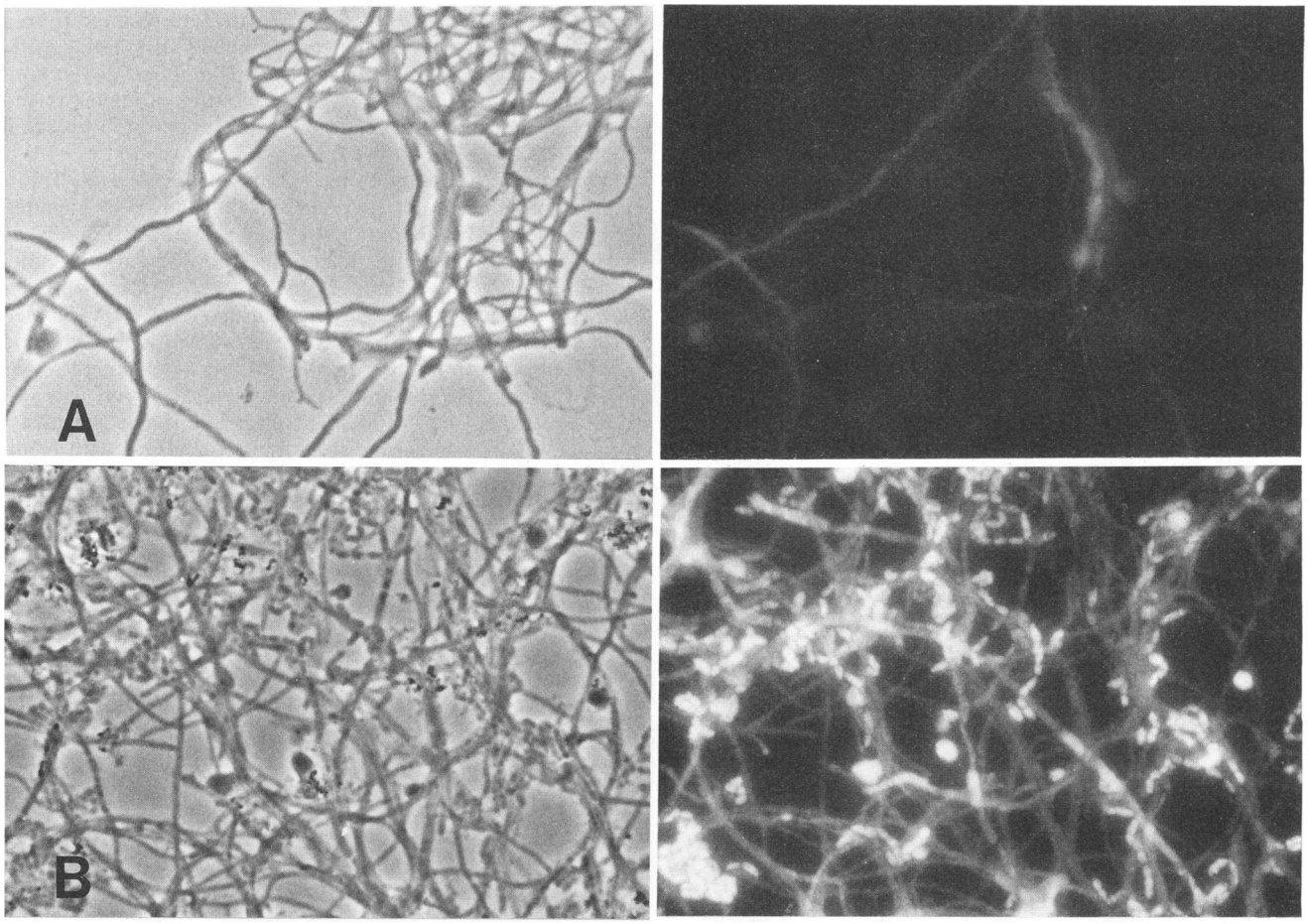

FIG. 2. Autofluorescence of vesicles and nonspecific binding of probes to vesicles reduced to the level of hyphae after lysozyme pretreatment, additional Nonidet P-40 and toluene treatments, and subsequent in situ hybridization with TRITC-labeled probe EFP (A, right panel). The hybridization signal intensity of *Frankia* sp. strain Ag45/Mut15 was quite low after in situ hybridization with TRITC-labeled universal probe Eub338 compared with the fast-growing background organism *L. lactis* subsp. *cremoris* (B). The left panels are the corresponding phase-contrast photographs.

therefore, attributed mainly to autofluorescence and to nonspecific binding of the oligonucleotide probes. Lysozyme treatment, as well as washing with a nonionic detergent (Nonidet P-40) or a solvent (toluene) with or without additional pretreatments, caused vesicles to enlarge and change their shape (Fig. 2A). This change of shape was possibly due to the removal of the multilaminate outer layer composed of monolipid layers which surrounded the vesicles (27, 32). Concomitantly, the level of autofluorescence of these vesicles decreased to the level of the filaments. The permeabilization of the vesicles, however, was not uniform. Only some of the vesicles were totally permeable; many vesicles remained in their original impermeable state and, at the same time, exhibited strong autofluorescence (Fig. 2B). Longer exposure to lysozyme and detergents could remove the outer layer of these vesicles as well, but at the same time this treatment would increase the risk of total lysis of other vesicles.

Treatments with Nonidet P-40 and toluene alone resulted in quite strong signals for both filaments and vesicles after hybridization with probes EFP and IFP. Strong signals were also obtained with eukaryotic probe Euk516. However, such nonspecific binding of the probe was not obtained after lysozyme treatment alone or in combination of this treatment with Nonidet P-40 or toluene or both, suggesting that this treatment made the cells more permeable to the probe.

Hybridization signals were also obtained with universal probe Eub338. In mixtures containing different bacteria, however, the signal intensity obtained with *Frankia* sp. was weak compared with the hybridization signal intensity obtained with fast-growing reference organisms, such as *L. lactis* subsp. *cremoris* (Fig. 2B).

Fluorescent probing of nodule homogenates. The results of the pretreatment studies done with pure cultures were not directly transferable to vesicles and vesicle clusters from nodules. These preparations seemed to be much more resistant to the pretreatments, and none of the treatments had any visible influence on vesicle size and shape or on autofluorescence.

Furthermore, the use of fluorescently labeled oligonucleotides to detect *Frankia* sp. in crushed nodules was hampered a great deal by autofluorescence. Plant material exhibited strong autofluorescence which made the detection of probe-conferred fluorescence at 580 nm (filter set 15) impossible. The autofluorescence signal of even small amounts of contaminating organic material was brighter than the hybrid-

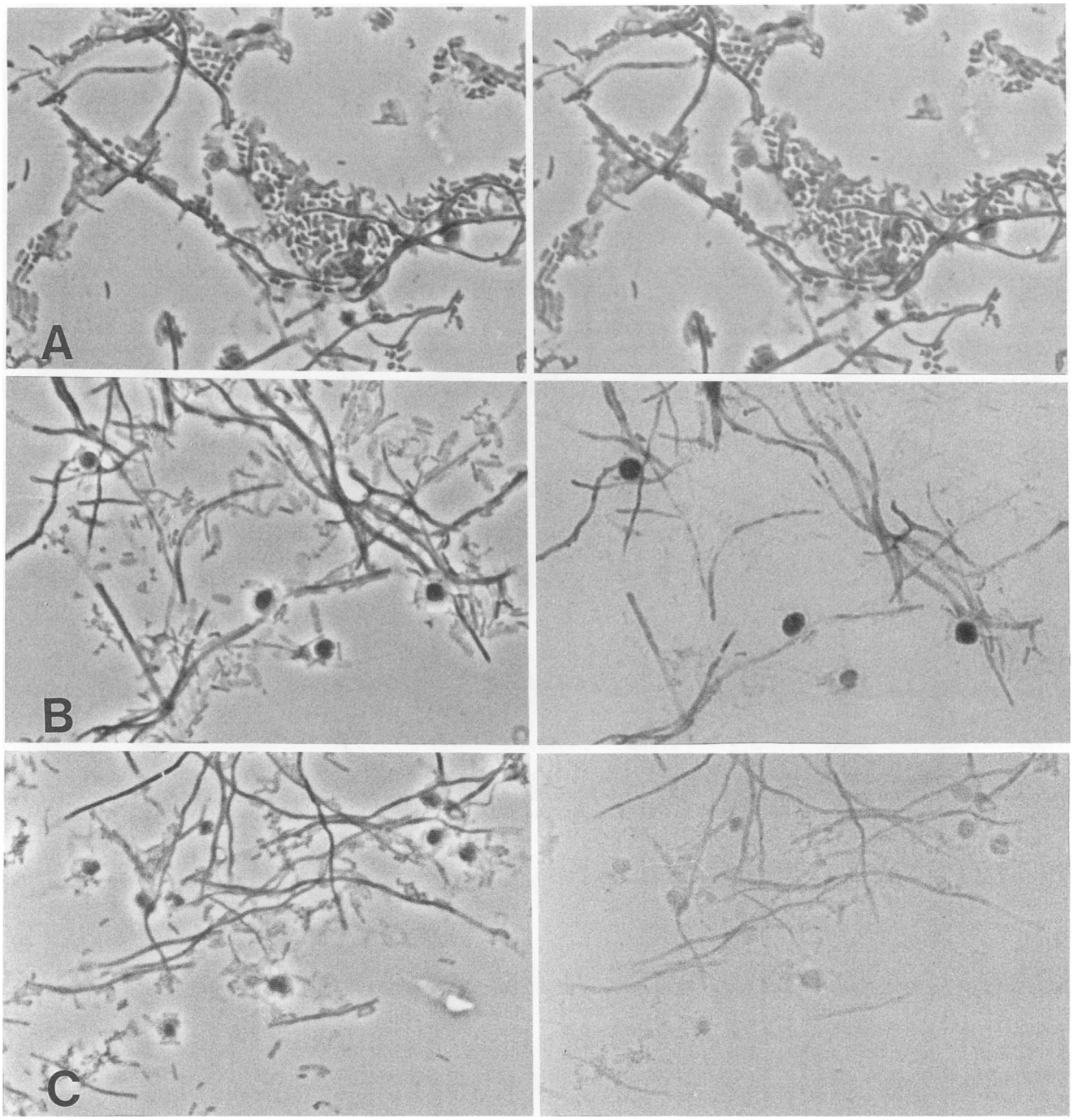

FIG. 3. Bright-field micrographs of *Frankia* sp. strain Ag45/Mut15 in a mixture of background organisms after in situ hybridization with digoxigenin-labeled oligonucleotides Eub338 (A), EFP (B), and Euk516 (C) and subsequent antibody-alkaline phosphatase detection (right panels). The left panels are the corresponding phase-contrast photographs.

ization signals obtained with *Frankia* sp. A nonfluorescence assay was therefore more likely to allow detection of *Frankia* sp. in crushed nodules.

Hybridization of *Frankia* sp. with digoxigenin-labeled probes. Digoxigenin-labeled oligonucleotide probes in combination with antibody-alkaline phosphatase detection allowed us to circumvent the problems of autofluorescence of the cell material itself, as well as the problem of contaminating material, and to increase the sensitivity of hybridization. Previous studies suggested that gram-positive cell walls were impermeable to antibody-alkaline phosphatase conjugates even after lysozyme treatment (40). Detection of *Frankia* sp. strain Ag45/Mut15 with digoxigenin-labeled probes EFP and Eub338 was therefore tried with additional pretreatments (Table 1).

Specific hybridization signals, however, were often

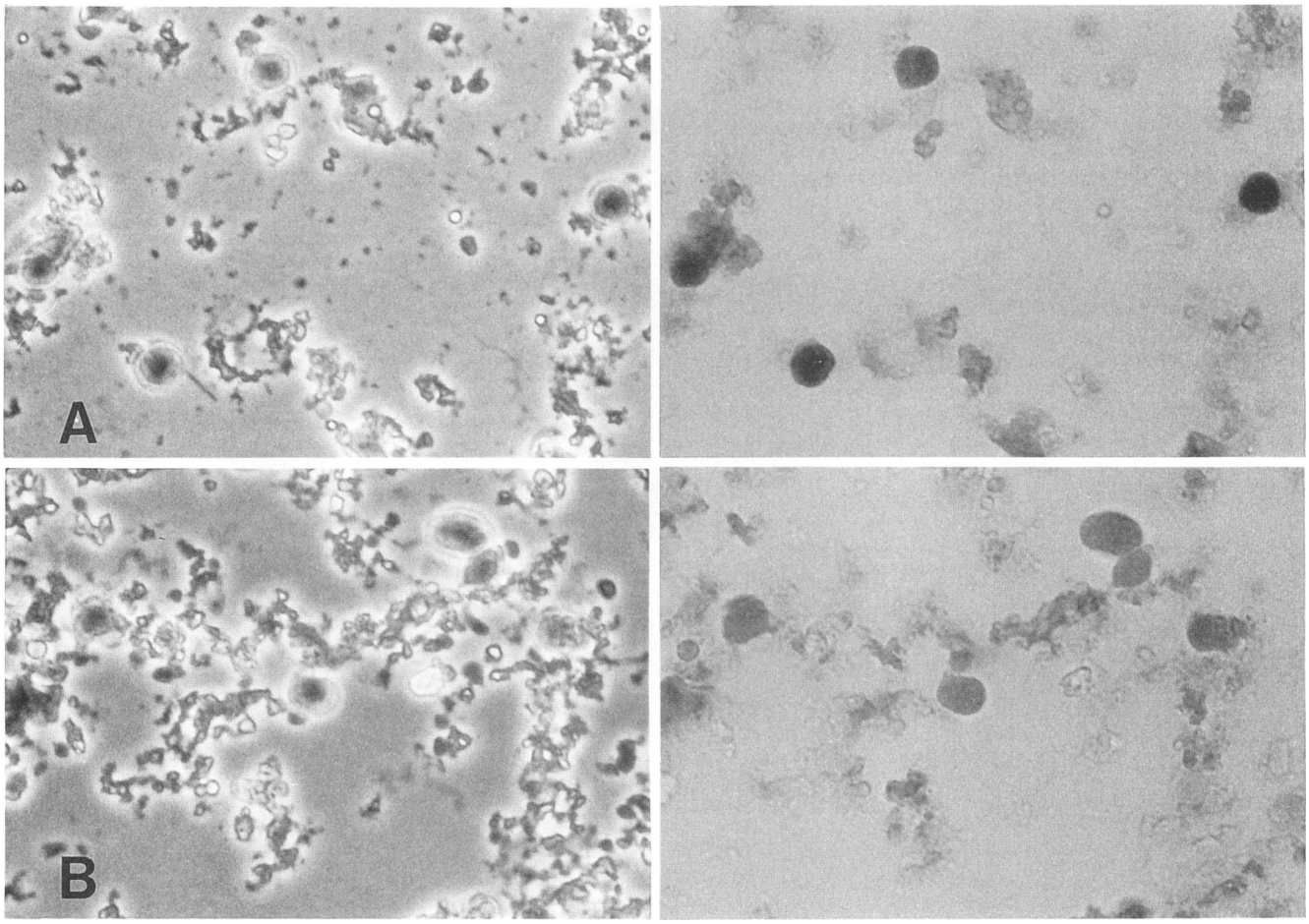

FIG. 4. Bright-field micrographs of *Frankia* vesicles in nodule homogenates after H_2O_2 treatment, subsequent lysozyme pretreatment, additional Nonidet P-40 and toluene treatments, in situ hybridization with digoxigenin-labeled oligonucleotides EFP (A) and Euk516 (B), and subsequent antibody-alkaline phosphatase detection (right panels). The left panels are the corresponding phase-contrast photographs.

masked by nonspecific binding of probes. Experiments with noncomplementary probes (IFP and Euk516) indicated that there was a high degree of nonspecific binding to both vesicles and filaments independent of the pretreatments. Probes appeared to bind specifically to *Frankia* cells, whereas background organisms were not stained. This nonspecific binding could be reduced by changing the buffer from the Tris-based hybridization buffer used with fluorescent probes to the maleic acid-based hybridization buffer. Nonspecific binding to filaments and vesicles after pretreatments with Nonidet P-40 or toluene alone, however, could not be eliminated by this change.

Although lysozyme pretreatment alone was sufficient for detecting rRNA sequences in vesicles, it was not adequate for reliable detection of rRNA in filaments. Additional treatments with Nonidet P-40, toluene, or both increased the signal-to-noise ratio and enhanced the reliability of the detection of filaments (Fig. 3). Hybridization with bacterial probe Eub338 revealed intense signals on both *Frankia* vesicles and filaments, as well as on background organisms (Fig. 3A). Hybridization with *Frankia*-specific probe EFP allowed specific detection of *Frankia* sp. strain Ag45/Mut15 among large numbers of background organisms (Fig. 3B). Filaments and vesicles were well stained and could be distinguished from the other microorganisms. In addition, the signal intensity of vesicles was much greater than the signal intensity of filaments. Detection of the digoxigenin reporter group on probe EFP by the antibody-alkaline phosphatase assay, however, supplied sufficient information concerning the specificity of hybridization even after a short incubation period (about 3 h). Longer incubation increased the signal intensity of both filaments and vesicles. After incubation for up to 16 h, nonspecific background staining became more pronounced; however, the increase was not significant. Hybridization with eukaryotic probe Euk516 revealed weak binding to *Frankia* cells, but not to background organisms (Fig. 3C).

Permeabilization of the hyphae could also be achieved by additional incubation with proteinase K after lysozyme treatment. However, proteinase K was quite difficult to use. Only very low concentrations of proteinase K (10 ng ml^{-1}) could be used for short incubation times (e.g., 10 min at room temperature). Higher concentrations either destroyed the cell material (e.g., 100 ng ml^{-1}) or resulted in the loss of target molecules from the cells so that no or only weak hybridization signals were obtained.

Identification of *Frankia* vesicles in nodule homogenates with digoxigenin-labeled probes. In situ hybridization with digoxigenin-labeled oligonucleotides EFP and Eub338 and subsequent enzymatic detection of the digoxigenin reporter

group in crushed nodules of *A. glutinosa* did not result in reliable hybridization signals. Pretreatments with Nonidet P-40, lysozyme, toluene, or proteinase K only occasionally revealed some vesicles showing hybridization signals. These results, however, were not reproducible. Thus, pretreatment studies were expanded in order to obtain reasonable permeability of the capsule surrounding the vesicles. Enzymatic digestion with cellulase, pectinase, or a combination of both enzymes for up to 4 h before lysozyme and detergent treatments had no influence on permeability. Peracetic acid treatment for 1 h before enzymatic digestion resulted in hybridization signals which were due to nonspecific binding of the probe. Longer exposure times reduced the amount of vesicles dramatically. Alternative pretreatment of the nodule homogenates with H_2O_2 (15% for 1 h) instead of peracetic acid resulted in specific hybridization signals on vesicles with or without additional cellulase or pectinase treatments (Fig. 4A). Detection of *Frankia* sp. in nodule homogenates was restricted to vesicles; no filaments or spores were detected in hybridization experiments. The signals obtained after hybridization with probe Euk516 did not differ from those obtained with the antibody-alkaline phosphatase conjugate alone (Fig. 4B). Subsequent permeabilization of the bacterial cells by lysozyme, Nonidet P-40, and toluene pretreatments, however, remained necessary for specific detection.

The technique described here should be a powerful tool in the identification of *Frankia* isolates, in the characterization of as-yet-uncultured nodule populations, and in the confirmation of the origin of unusual isolates. However, many typical *Frankia* strains which form vesicles in nodules and which belong to the same compatibility group have identical 16S rRNA sequences and therefore cannot be differentiated by rRNA-targeted hybridization (20, 31). This problem of limited specificity of 16S rRNA-targeted oligonucleotide probes for strains or isolates can probably be solved by targeting a highly variable insertion on 23S rRNA which exhibits great sequence differences even between closely related *Frankia* strains (35).

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