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Detection of mRNA in *Streptomyces* Cells by Whole-Cell Hybridization with Digoxigenin-Labeled Probes

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Detection of mRNA of the thiostrepton resistance gene (*tsr*) harbored by plasmid pIJ673 in *Streptomyces violacelatus* was achieved by whole-cell hybridization with digoxigenin-labeled in vitro transcripts followed by an antibody-alkaline phosphatase detection of the digoxigenin reporter molecule. Prior to hybridization, the cells had to be permeabilized by lysozyme, the detergent Nonidet P-40, and toluene. The permeability of the *S. violacelatus* cells for probes and the antibody-alkaline phosphatase conjugate was demonstrated by hybridization with digoxigenin-labeled, 16S rRNA-targeted oligonucleotides.

Streptomycetes are gram-positive soil bacteria capable of synthesizing a large number of secondary metabolites with antibacterial or antifungal activity (12). However, although the inhibitory effects of these metabolites can be easily demonstrated in defined artificial systems, there is very limited knowledge of the significance of these antibiotic substances in natural environments (28). Studies of the distribution and occurrence of antibiotic-producing organisms and their activity in natural habitats have been largely hampered by the lack of sensitive methods for detection. Modern molecular techniques that are based on the detection of rRNA or mRNA in whole cells by labeled complementary probes may offer opportunities to address questions of the in situ detection of bacteria and secondary metabolite production.

In situ or whole-cell hybridization techniques are widely used to detect and localize specific nucleic acids in tissues and cells. The quite laborious method with probes labeled with radioisotopes for these hybridization experiments (14, 22, 23, 26, 29) has stimulated the search for alternative labeling strategies. Whole-cell hybridization with rRNA-targeted oligonucleotides coupled to fluorescent dyes has found a wide application in the detection and identification of bacteria (1–4, 10, 11, 13, 15). The limited sensitivity of directly labeled fluorescent probes, however, has led to a second indirect strategy in which reporter molecules like biotin or digoxigenin are attached to the probes. The formation of stable hybrids is shown by labeled binding proteins (e.g., avidin, streptavidin, or antibodies) used to detect these reporters. The sensitivity of these detection protocols has reached a level comparable to that of methods based on radioisotopes (5, 24). The digoxigenin reporter molecule has been used in rRNA-targeted oligonucleotide probes, polymerase chain reaction amplification products, or in vitro transcripts in the detection and identification of single bacterial cells (30), in the detection of genes on the chromosome (7), or in the localization of RNA in transections, respectively (6, 17, 27). However, in bacteria the efficiency of the whole-cell hybridization strategy is reduced since cell walls and cell membranes form penetration barriers for probes (15) and for the antibody-enzyme conjugate (30). In many gram-negative bacteria, pretreatments with lysozyme facilitate reliable detection of the digoxigenin reporter molecule by the antibody-enzyme conjugate (30). For gram-positive bacteria, however, these pretreatments are not sufficient to enable the antibody-enzyme conjugate to penetrate.

Streptomyces violacelatus (ISP 5438) harboring plasmid pIJ673, a derivative of the multicopy plasmid pIJ101 (20), was used to optimize the digoxigenin-based whole-cell hybridization technique for a gram-positive bacterium. Plasmid pIJ673 contains the thiostrepton resistance gene (*tsr*) which codes for a constitutively produced 23S rRNA methylase (9, 25). In this case, not only the rRNA but also the *tsr* mRNA transcribed in high numbers from pIJ673 could serve as a potential target. Spores of strain ISP 5438 were inoculated into Tryptone soy broth (Difco) containing 10% sucrose and 5% polyethylene glycol 6000 and aerobically cultivated with or without thiostrepton (50 μg ml^{-1}; Sigma) for 1, 2, and 4 days on a shaker at 200 rpm at 30°C. *S. violacelatus* cells without plasmid pIJ673 were grown under the same conditions without thiostrepton. Mycelium was harvested by centrifugation, washed with phosphate-buffered saline (PBS; composed of 0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 [pH 7.2]) and fixed in 3 volumes of fixation buffer (composed of 4% paraformaldehyde in PBS) at 4°C for 3 to 16 h. Subsequently, the cells were washed once in PBS and stored in ethanol-PBS (1:1 [vol/vol]) at −20°C until further use (2).

The presence or absence of mRNA of the *tsr* gene in fixed cells was confirmed by dot blot and Northern (RNA) blot analysis of total RNA extracts (21). For RNA extraction, fixed cells stored in ethanol-PBS were washed in PBS and incubated with lysozyme (1 mg ml^{-1} in 100 mM Tris–50 mM EDTA [pH 7.5]) at 37°C for 30 min. Complete cell lysis was achieved by the addition of sodium dodecyl sulfate (SDS) (to 0.1%) and proteinase K (to 200 μg ml^{-1}) and incubation at 37°C for another 30 min. Purification of total RNA was performed in accordance with the method of Hughes and Galau (19). The presence of RNA was confirmed by gel electrophoresis and ethidium bromide staining. RNA samples (20 μg, based on the optical density reading at 260 nm) were applied to nylon membranes (Magnagraph; Kontron, Schlieren, Switzerland) and hybridized in accordance wtih

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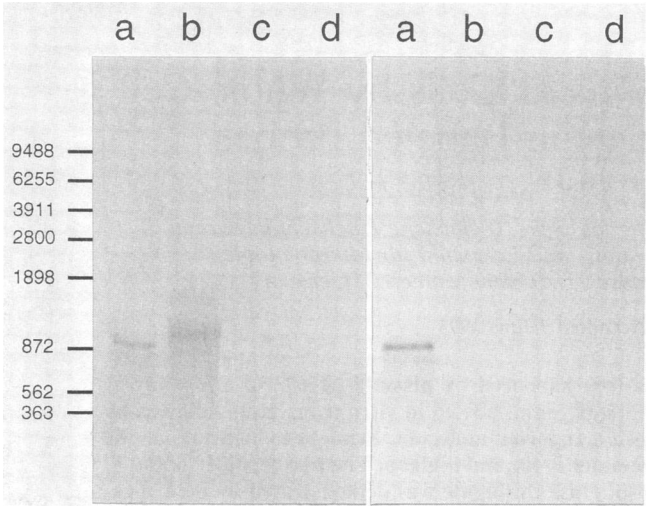

FIG. 1. Northern blot hybridization demonstrates binding of digoxigenin-labeled antisense probe (left panel) to RNA isolated from cells harboring plasmid pIJ673 (lane b). RNA of cells without plasmid (lane c) and RNase-treated samples (lane d) do not exhibit hybridization signals. Hybridization with the sense probe (right panel) does not show signals obtained with the antisense probe. The digoxigenin-labeled in vitro transcript (lane a) serves as a size standard for approximately 0.9 kb. An external size standard is given by an RNA marker (Promega), with sizes (nucleotides) indicated on the left.

the method of Church and Gilbert (8), with digoxigenin-labeled in vitro transcripts of the *tsr* gene as probes.

For in vitro transcription, an 800-bp *tsr* gene fragment (*Cla*I-*Bcl*I; Promega) from plasmid pIJ487 (18) was cloned into pGEM-7zf (Promega), and sense and antisense RNA probes were transcribed after linearization of the plasmid (*Cla*I or *Sac*I) with either T7 RNA polymerase (Promega) or SP6 RNA polymerase (Pharmacia) and digoxigenin-UTP (Boehringer) in accordance with the manufacturers' instructions. The probes were partially degraded to an average size of 200 nucleotides by heating at 60°C for 90 min in a buffer containing 0.2 M Na_2CO_3 and 0.2 M $NaHCO_3$ (23).

The labeling efficiency was tested on identical amounts of spotted probes. Both probes showed a comparable signal intensity after antibody-alkaline phosphatase detection. Dot blot hybridization as well as Northern blot hybridization showed specific hybridization signals with the digoxigenin-labeled antisense probe, whereas no signals were obtained with the sense probe. Northern blot analysis, performed at a 55°C hybridization temperature in the presence of 50% formamide, showed a dominant band which was somewhat larger than the labeled in vitro transcript (about 0.9 kb) in cells grown in the presence of thiostrepton, whereas no signal was obtained with RNA of the strain without plasmid or with RNase-treated samples containing the plasmid (Fig. 1). Hybridization with the sense probe did not show this band. These results indicate the presence of mRNA of the *tsr* gene in fixed cell material of *S. violacelatus* harboring plasmid pIJ673.

Prior to their application to slides for whole-cell hybridization, the *Streptomyces* cells were sonicated mildly to disrupt cell clumps. One microliter was applied to gelatin-coated slides [0.1% gelatin, 0.01% $KCr(SO_4)_2$] and allowed to air dry. Following dehydration in 50, 80, and 96% ethanol for 3 min each, the preparations were incubated with lysozyme (1 mg ml^{-1} in 100 mM Tris–50 mM EDTA [pH 7.5]) at room temperature for 30 min and afterwards washed in 100 mM Tris (pH 7.5)–10 mM EDTA supplemented with 0.1% Nonidet P-40 (Sigma) at room temperature for 10 min. After dehydration in 50 and 80% ethanol, the slides were washed in toluene (1% in 96% ethanol) and 96% ethanol for 3 min each and finally air dried.

To demonstrate the permeability of the cells for probes and the antibody-alkaline phosphatase conjugate, the samples were hybridized with digoxigenin-labeled, 16S rRNA-targeted oligonucleotides. The universal eubacterial probe Eub338 (1), the *Frankia*-specific probe EFP (16), and the eukaryotic probe Euk516 (1), respectively, were labeled with digoxigenin-ddUTP (Boehringer) at the 3′ end with terminal transferase (Promega) in accordance with the manufacturers' instructions. The hybridization was performed in 7 μl of hybridization buffer (0.9 M NaCl, 100 mM Tris, 10 mM EDTA, 0.01% SDS [pH 7.4]) and 1 μl of probe (25 ng μl^{-1}) at 42°C for 2 h.

After hybridization, the slides were washed in hybridization buffer at 45°C for 20 min and subsequently incubated with 10 μl of alkaline phosphatase-conjugated anti-digoxigenin F_{ab} fragments (Boehringer) diluted 1:10 in 150 mM NaCl–100 mM Tris-HCl (pH 7.5)–0.5% blocking reagent (Boehringer) at 27°C for 1 h (30). Alkaline phosphatase was visualized by formation of a water-insoluble dark precipitate after incubation in 50 μl of substrate solution (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate [Boehringer] in 100 mM Tris-HCl–100 mM NaCl–50 mM $MgCl_2$ [pH 9.5] in accordance with the manufacturers' instructions) at room temperature for 3 to 16 h. Afterwards, the slides were rinsed with distilled water and air dried, and the preparations were examined with a microscope (Zeiss, Oberkochen, Germany).

After whole-cell hybridization with 16S rRNA-targeted oligonucleotides of untreated or incompletely pretreated hyphae, i.e., Nonidet P-40 or toluene pretreatment only, most cells remained unstained and only a few revealed some staining (Fig. 2a). This partial staining was also obtained by applying the antibody-alkaline phosphatase treatment without a previous hybridization, and it could be removed by lysozyme pretreatment. It is suggested therefore that these signals were caused by cell wall components of the organism itself. When hyphae were pretreated with lysozyme, Nonidet P-40, and toluene, the permeability of the cells allowed the penetration of the probe and the antibody-enzyme conjugate. *S. violacelatus* cells harvested after 1, 2, or 4 days could be detected after hybridization with the universal eubacterial probe (Fig. 2b). Hybridization with probe EFP, which hybridized to *Frankia* sp. strain Ag45/Mut15 or the eukaryotic probe Euk516 did not result in staining (Fig. 2c). Nonspecific binding of these probes or of the antibody-enzyme conjugate to hyphae was not observed.

After a good labeling efficiency of the *tsr* mRNA-targeted in vitro transcripts and a sufficient permeability of the cells for the antibody-enzyme conjugate were demonstrated, detection of the *tsr* mRNA in *S. violacelatus* cells was attempted. Because of the less-frequent occurrence of transcript targets compared with rRNA, this could be achieved only by using probes with sensitivity higher than that of end-labeled oligonucleotides. In vitro transcripts targeted to the *tsr* mRNA were therefore used as a specific polynucleotide probe carrying multiple labels (17). Whole-cell hybridizations with transcript probes were performed in 7 μl of 50% formamide in hybridization buffer and 1 μl of probe (approximately 20 ng) at 45°C for 16 h. Conditions for

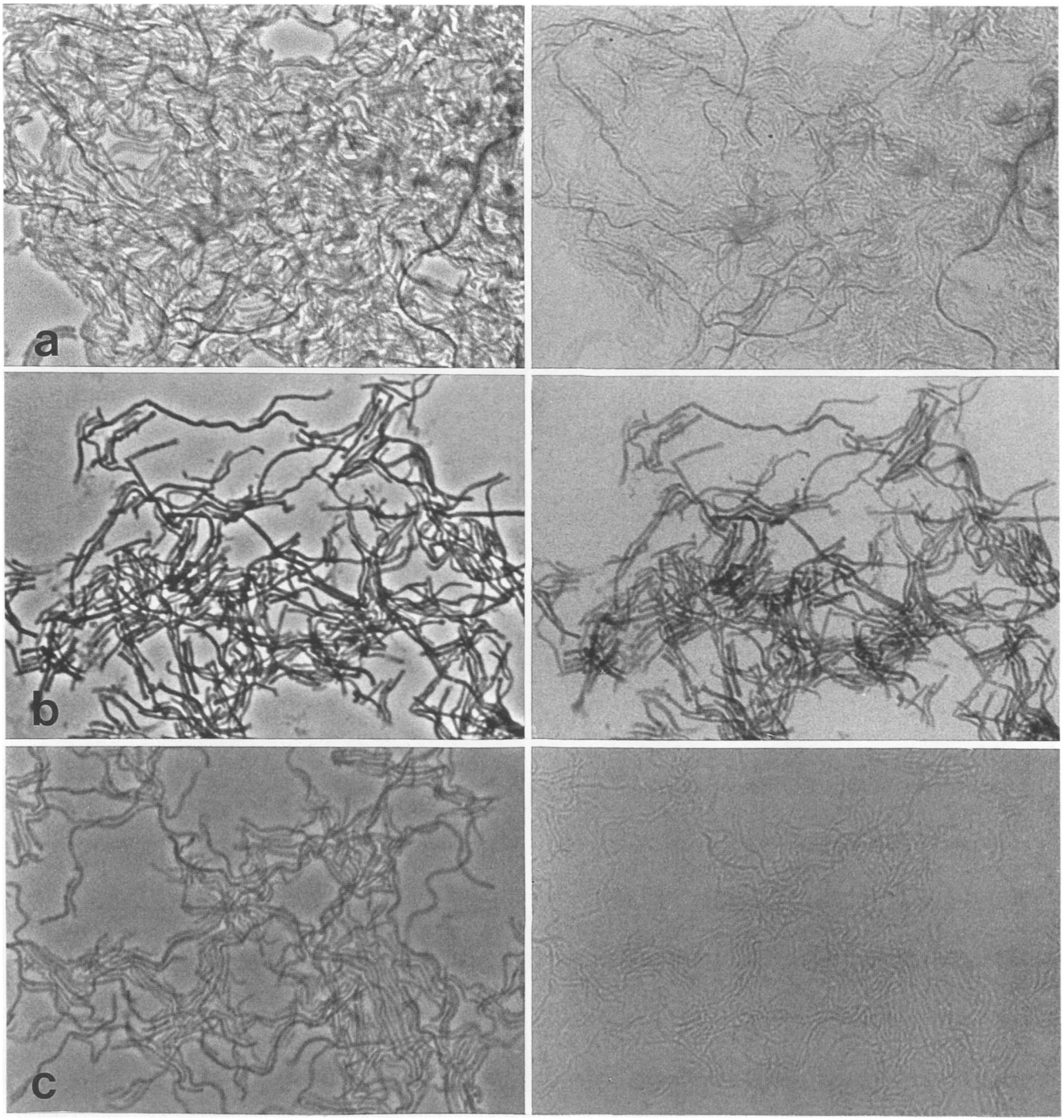

FIG. 2. Phase-contrast (left) and bright-field (right; visualizing only stained cells) micrographs of *S. violacelatus* grown in the presence of thiostrepton for 4 days. In situ hybridization with the digoxigenin-labeled, 16S rRNA-targeted eubacterial probe Eub338 demonstrates that all cells are permeable for the probe and the antibody-enzyme conjugate (b, right). The nonbinding probe EFP does not show any hybridization signals (c, right). Non-pretreated cells showed some staining after in situ hybridization (a, right). These signals were also obtained without hybridization or other treatments and could be removed by lysozyme treatment.

washing and detection were the same as those in hybridizations with oligonucleotide probes. Hybridization with the digoxigenin-labeled antisense probe and subsequent antibody-alkaline phosphatase detection of the reporter molecule on hyphae of *S. violacelatus* harboring plasmid pIJ673 revealed distinct signals in all hyphae (Fig. 3a and b), whereas hybridization with the sense probe did not exhibit any signals (Fig. 3c). *S. violacelatus* without plasmid did not

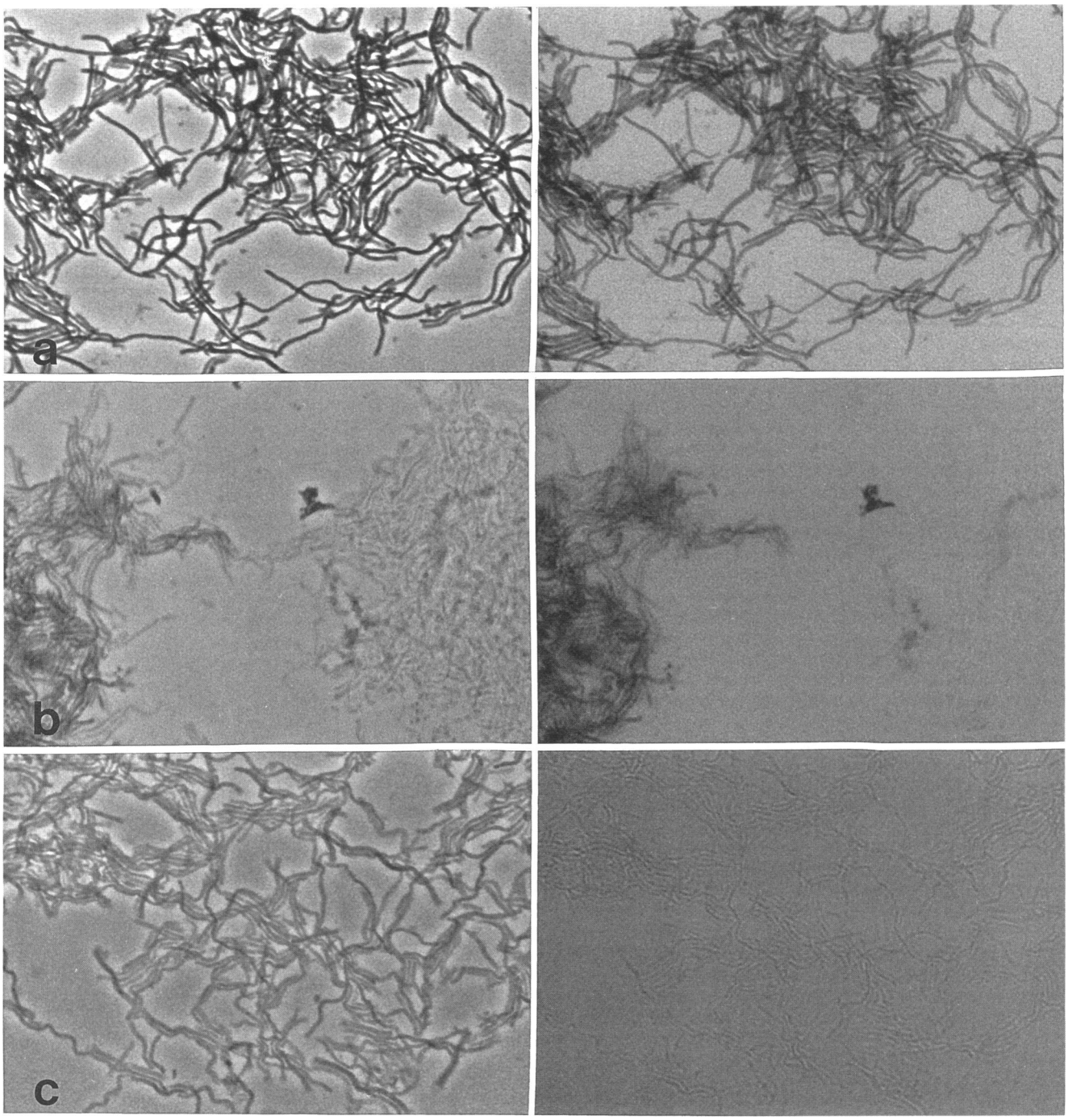

FIG. 3. Bright-field micrograph of *S. violacelatus* grown in the presence of thiostrepton for 2 days (a, right) showing distinct signals in hyphae after in situ hybridization with the digoxigenin-labeled antisense probe and subsequent antibody-alkaline phosphatase detection of the reporter molecule. *S. violacelatus* without plasmid does not show hybridization signals in a mixture with cells harboring the plasmid (b, right). Concomitant hybridization with the sense probe does not show hybridization signals (c, right). The left panels show the corresponding phase-contrast photographs.

show hybridization signals (Fig. 3b). Additional hybridization experiments with sense and antisense transcripts of *nifH* from *Frankia* species used as the control for nonspecific binding of the probes to *S. violacelatus* did not reveal any signals indicating that the detection of the *tsr* transcript was specific. A quantitative evaluation of the signals, however, is not yet possible since differences in signal expression could be due to either different amounts of target sequences per cell or differences in cell permeability. This report demonstrates that a qualitative detection of transcripts in *Strepto-*

myces cells is possible. The described approach of detecting mRNA in fixed whole cells of bacteria should facilitate in situ studies of gene transcription and thereby indirectly facilitate studies of specific activities in individual cells.

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