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Direct Detection of Recombinant Gene Expression by Two Genetically Engineered Yeasts in Soil on the Transcriptional and Translational Levels

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The expression of a recombinant gene by yeasts seeded into soil samples was directly measured by analyzing transcripts and gene product occurrences in soil extracts. Two yeast species, *Saccharomyces cerevisiae* WHL292 and *Hansenula polymorpha* LR9-Apr4, both engineered by a synthetic gene sequence encoding the mammalian peptide aprotinin, produced and secreted this peptide in batch cultures at concentrations of 90 and 64 ng ml^{-1}, respectively. In *S. cerevisiae*, the aprotinin gene was located on plasmid p707 and expressed constitutively. *H. polymorpha* carried the gene chromosomally integrated, and its expression was inducible by methanol. To detect aprotinin transcripts, cells were directly lysed in the soil samples and the crude lysates were hybridized to oligo(dT)-coated magnetized polystyrene beads (Dynabeads). After separation and purification in a magnetic field, aprotinin mRNA was detected by reverse transcriptase PCR with aprotinin gene-specific primers. Transcripts from 10 cells g of soil^{-1} were sufficient for detection. When 10^7 cells of *S. cerevisiae* were inoculated into soil, aprotinin mRNA was detectable during the first 4 days. Addition of methanol and a combined nutrient solution was necessary to induce aprotinin gene expression of *H. polymorpha* in soil. Aprotinin could be detected directly in soil extracts by an indirect enzyme-linked immunosorbent assay with monoclonal aprotinin-specific antibodies. The detection threshold was 45 pg g of soil^{-1}. In presterilized soil inoculated with *S. cerevisiae* (10^6 CFU g^{-1}), aprotinin accumulated during the first 10 days to 12 ng g of soil^{-1} and then remained constant. In nonsterile soil, however, aprotinin formation was observed only during the first 4 days and was followed by a decrease below the level of detection after 2 weeks. Similar results were obtained with induced cells of *H. polymorpha*. Thus, disappearance of aprotinin was due to its degradation by the indigenous soil microorganisms.

The release of genetically engineered microorganisms into the environment is often directly connected to the question whether the recombinant traits are actually expressed under in situ conditions. In cases of deliberate releases, e.g., of biodegradative or plant growth-promoting rhizobacteria, the monitoring of recombinant gene expression would quickly indicate whether the organisms perform their activity as desired. Unintended releases of genetically engineered microorganisms, e.g., from fermentation plants, need to be evaluated for their ecological risk. Gene expression data could help determine if survival of a certain genetically engineered microorganism poses a risk to the environment because of the introduction of an altered phenotype (35).

The expression of genes involves their transcription from DNA into mRNA followed by their translation from mRNA into protein. The specific detection of both transcripts (mRNA) and gene products (proteins) can be used to study gene regulation and activity of microorganisms. The analysis of bacterial transcripts is difficult because mRNA half-life values generally range only from 0.5 to 10 min (27). In eukaryotic cells, however, mRNAs undergo various co- and posttranscriptional modifications such as capping of the 5′ end, polyadenylation of the 3′ end, or internal elimination of sequences by splicing (7). As a result, the transcript stabilities can increase to several hours. If DNA sequence information is available, specific and highly sensitive tools such as nucleic acid hybridizations or reverse transcriptase (RT) PCR are available to study environmental gene expression.

Protocols for the direct extraction of mRNA have been developed to study the expression of prokaryotic genes in marine environments (20, 29, 30), freshwater (26), and soil (9, 36). Specific detection of transcripts in these reports has been restricted to nucleic acid hybridizations. For positive detection signals, approximately 10^6 bacterial cells were necessary. In aquatic systems, different filtration techniques can be applied to concentrate cells, and thus, detection thresholds of 10^4 cells per ml of seawater or slightly less can be accomplished. In soil, however, humic acids are coextracted with RNA (9, 36). These compounds are known to interfere with nucleic acid detection methods such as hybridizations and PCR (17, 33, 34). Together with the instability of prokaryotic mRNA, quantitative transcript analysis in soil is still relatively insensitive with detection thresholds of 10^6 to 10^9 cells per g of soil (9, 28, 36). The inclusion of an RT step converting the mRNA strand into a DNA strand followed by PCR amplification has a high potential to increase detection sensitivities and even be applied for future quantitative gene expression studies (1, 25).

The detection of proteins can generally be accomplished with specific monoclonal or polyclonal antibodies (2). With enzyme-linked immunosorbent assay (ELISA) techniques, high-

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TABLE 1. Characterization of the yeast strains used in this study

Yeast strain	Localization of the aprotinin gene	Promoter	Expression	Source
Saccharomyces cerevisiae WHL92	Plasmid p707	MFα	Constitutive	H. Wehlmann
Hansenula polymorpha LR9	Integrated with four tandem repeats of pFMD130 with an aprotinin gene sequence inserted into the chromosome	FMD	Methanol inducible	G. Gellissen

ly sensitive detections are possible (38). Environmental applications of ELISA techniques with specific regard to terrestrial systems have mainly focused on the detection of pesticides (15, 16, 18) or viruses (12) or the study of the occurrence, survival, or competition of microorganisms (24, 37). However, if specific antibodies are available, the technique has a high potential for the sensitive detection of specific gene products in soil. In the case of recombinant gene products, which normally do not occur in a specific microbial habitat such as soil, the gene expression of introduced microorganisms should directly be detectable after protein extractions.

The intention of this investigation was to determine whether genetically engineered microorganisms designed to produce a pharmaceutically relevant compound in fermentations are able to express their recombinant trait in soil. For the production of heterologous proteins, especially those designed for human therapeutical applications, yeast strains are the preferred microorganisms. In contrast to prokaryotic microorganisms such as *Escherichia coli*, the eukaryotic yeasts are more likely to produce properly folded proteins and are able to perform further protein modifications such as glycosylation (14). As model organisms, we selected two yeast strains carrying the same recombinant structural gene. The sequence of this gene was derived from the amino acid sequence of the mammalian peptide aprotinin (10). One strain, *Saccharomyces cerevisiae* WHL292, carried the gene on a plasmid and synthesized aprotinin constitutively. The other strain, *Hansenula polymorpha* LR9-Apr4, carried the gene in four tandem repeats chromosomally integrated. In this strain, the expression was under the control of a formate dehydrogenase (FMD) promoter and thus was methanol inducible. Gene expression of both strains was monitored in seeded soil samples incubated for up to 2 weeks. Total eukaryotic mRNA from cells directly lysed in soil suspensions was extracted by hybridizing the poly(A) 3′ end of the mRNA to oligo(dT)-coated magnetized polystyrene particles (Dynabeads). The aprotinin transcripts were detected by RT-PCR. Aprotinin formation was also detected with an indirect ELISA technique by hybridizing aprotinin-specific monoclonal antibodies to peptide-containing soil extracts.

MATERIALS AND METHODS

Microorganisms. Two yeast strains, *S. cerevisiae* WHL292 p707 and *H. polymorpha* LR9-Apr4, both genetically engineered to produce the peptide aprotinin (10), were selected for this study (Table 1). The aprotinin gene sequence (174 bp) was designed and synthesized by Bayer AG, Wuppertal, Germany. In *S. cerevisiae* WHL292, the aprotinin gene was located on plasmid p707 (8.4 kb), which is a derivative of pJS212 (8). The aprotinin gene was constitutively expressed by the MFα-factor promoter (4) in this strain. Plasmid p707 was also maintained in *E. coli* HB101. Both strains were provided by H. Wehlmann, Wuppertal, Germany. *H. polymorpha* LR9-Apr4 contained the sequence of plasmid pFMD130, including the aprotinin insert flanked by an α-leader gene and a terminator sequence (4, 39), chromosomally integrated with four tandem repeats (13). The aprotinin gene expression was regulated by the FMD promoter and thus under pure culture conditions was repressed by glucose and inducible by methanol (13). The strain was obtained from G. Gellissen, Düsseldorf, Germany.

Cultivations. *S. cerevisiae* WHL292 p707 was cultivated in SD medium (pH 6.0) (32). *H. polymorpha* LR9-Apr4 was cultivated in malt medium (10 g of malt extract [Difco Laboratories, Detroit, Mich.] and 3 g of peptone [Difco] per liter [pH 5.6]). Solid media were prepared by the addition of agar (1.5%, technical grade [Oxoid, Unipath Ltd., Basingstoke, Hampshire, England]). The yeasts were cultivated at 30°C, and broth cultures were grown in a shaking incubator (TM-1; Infors AG, Basel, Switzerland) at 250 rpm. Aprotinin synthesis of *H. polymorpha* LR9-Apr4 was induced by the addition of methanol (final concentration, 1 volume %).

Soil. The soil was a phaeozem clay silt (8.0% sand, 72.2% silt mineral, 19.8% clay material), pH 7.1, with 1.97% organic carbon, 0.195% total organic nitrogen, and a total water-holding capacity (WHC) of 27.8%. The soil was collected in spring from the plow layer of a field near Jerxheim (Lower Saxony, Germany). The soil was air dried at room temperature to 10 to 20% of the WHC, sieved (2-mm mesh size), and then stored at 4°C in the dark for up to 6 months.

Soil inoculation, induction of aprotinin synthesis, and incubation. All experiments were carried out in triplicate. Soil samples were distributed in portions of 5 g into polypropylene test tubes (50-ml Falcon tubes; Becton Dickinson, Paramus, N.J.). For sterilization, soil was autoclaved for 1 h at 121°C and humidity was readjusted to 10 to 20% of the WHC. Soil samples were preincubated at 20°C overnight. Yeast cells, grown in batch cultures to late log growth phase, were harvested by centrifugation (10 min at 5,000 × *g*) and resuspended in aqueous 0.85% NaCl solution. The cell concentrations were determined microscopically with a counting chamber and diluted with 0.85% NaCl solution to the final inoculum concentration. Each tube was then inoculated by applying 250 to 500 μl of the respective cell suspensions dropwise onto the surface of the soil samples to result in a 50% saturation of the WHC. The inoculated soil samples were incubated at 20°C in the dark.

The threshold of detection for gene expression was determined by the addition of pure culture cells grown to late log phase in batch fermentations. Soils were inoculated with defined numbers of cells and incubated for 3.5 h at 22°C before mRNA extraction.

To induce *H. polymorpha* LR9-Apr4 in soil, methanol was applied onto the soil surface of inoculated samples. The concentration of methanol was 3.5% in the soil water. In some induction experiments, a combined nutrient solution (pH 5.6), consisting of yeast extract (3 g liter^{-1}), malt extract (3 g liter^{-1}), and peptone (5 g liter^{-1}), was supplied with the methanol solution. Inductions were performed 3.5 h before mRNA or aprotinin peptide extractions.

Extraction and isolation of yeast cells from soil. Soil samples were suspended in 9 volumes of 0.1% sodium hexametaphosphate solution, first by vortexing at 2,500 rpm for 30 s (VF2; Janke & Kunkel, IKA Labortechnik, Staufen, Germany) and then on an overhead shaking incubator at 220 rpm in the cold room (4°C) for 30 min. The resulting suspensions were diluted in 0.85% NaCl, and appropriate dilutions were cultivated in the growth media mentioned above. For experiments with unsterilized soil, the media were amended with ampicillin (50 mg ml^{-1}) and chloramphenicol (100 mg ml^{-1}) to inhibit bacterial growth.

Gene probe. The aprotinin gene probe was prepared by PCR. Template DNA was plasmid p707 extracted from *E. coli* HB101 according to the method of Birnboim (3). PCRs were carried out essentially as described by the manufacturer (Boehringer Manual digoxigenin [DIG] Luminescent Detection Kit; Boehringer, Mannheim, Germany). Aprotinin gene-specific primers were Alf1 (5′ CGC AGC ATC CTC CGC ATT AG 3′), which derived from the α-leader sequence of the coding DNA strand, and Apr7 (5′ AGC ACC ACC GCA AGT ACG CA 3′), which represented the end of the aprotinin gene of the noncoding strand. The PCR was carried out in a 100-μl volume containing template DNA, 50 pmol of each primer, 0.1 mM deoxynucleotide triphosphates (Promega, Heidelberg, Germany), 0.02 mM DIG-UTP (Boehringer), 2.5 mM $MgCl_2$, and 4 to 5 U of *Taq* DNA polymerase (Boehringer). The amplifications were performed with a DNA thermal cycler (Omnigene TR3 CM220; Hybaid Ltd., Teddington, United Kingdom). The incubation was 94°C for 3 min; 30 cycles at 94°C for 1 min, at 72°C for 1 min, and at 50°C for 1 min; and a final cycle at 72°C for 10 min. After the reaction, 1 volume of ethanol was added for DNA precipitation and the tubes were incubated for 30 min at −20°C. The probes were then centrifuged for 45 min at 28,000 × *g* at 4°C. The pellet was washed with 70% ethanol, dried, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

Cell lysis, colony hybridization, and aprotinin gene detection. In experiments with nonpresterilized soil, the presence of the aprotinin gene was confirmed by colony hybridization of soil-reisolated yeast cells and an aprotinin gene probe. Colonies grown for 3 to 5 days on agar plates were transferred onto nylon membranes (Hybond N+; Amersham, Braunschweig, Germany). Additionally, two labelled corners of the filter were inoculated with appropriate yeast cells as positive and negative controls. The following incubations were performed by placing the nylon membranes onto filter papers, which were located on trays carrying different solutions. First, the filters were incubated for 25 min in a lysis

solution (1,000 U of lyticase [Sigma Chemical Co., Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany]) at 37°C and then for 10 min at room temperature in 10% sodium dodecyl sulfate (SDS). The nylon membranes were transferred onto filter paper with denaturing solution (1.5 M NaCl–0.5 M NaOH; 20 min at room temperature) and then incubated for 10 min in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA [pH 8.0]). The membranes were then placed onto dry filter paper, and a 2× SSC solution (diluted from a 20× SSC stock solution) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (31) was successively applied onto the membranes. Finally, the membranes were incubated for 30 min at 120°C in a dry oven and then stored at room temperature in the dark until colony hybridization was performed.

Hybridization of the membrane-bound DNA with the DIG-labelled gene probe was performed as described by the manufacturer (DIG detection kit, Boehringer). Hybridized products were detected by autoradiography (Kodak X-Omat AR; Eastman Kodak, Rochester, N.Y.).

Extraction and purification of eukaryotic mRNA from soil. All solid materials which came in contact with the extracted mRNA were rinsed with 0.1% diethylpyrocarbonate to inactivate nucleases. Remaining diethylpyrocarbonate was destroyed either by autoclaving at 121°C or in a dry oven at 120°C (31). To extract mRNA of the inoculated yeast strains from soil, 15 ml of lysis solution (0.5 M LiCl, 1% [wt/vol] lithium dodecyl sulfate, 5 mM dithiothreitol, 10 mM Na_2EDTA, 0.1 M Tris-HCl [pH 8.0]) and 0.4 ml of antifoam A (Sigma Chemical Co.) were pipetted into the polypropylene tubes (50 ml; Falcon; Becton Dickinson) which contained the soil samples (5 g). The samples were vortexed for 2 min at the highest setting (2,500 rpm; VF2). The suspension was then transferred with Pasteur pipettes into presterilized agate beakers (50 ml; Retsch, Haan, Germany) containing three agate balls and ground for 10 min in a mortar mill (Type S1, Retsch) at maximum speed. The suspension was transferred to prechilled polypropylene tubes (50 ml) which were placed on ice. A suspension of 0.5 ml of oligo(dT) Dynabead solution (Dynal, Oslo, Norway), corresponding to 2.5 mg of oligo(dT)-coated Dynabeads, was added. The tubes were transferred to a shaking incubator (40 rpm at room temperature for 15 min) to allow hybridization between the oligo(dT)-coated Dynabeads and the poly(A) 3′ end of the eukaryotic mRNA. A titration column (length, 36 cm; total volume, 30 ml; outer diameter, 0.9 cm), which was placed in a magnetic field, was used to separate the mRNA-loaded oligo(dT) Dynabeads from the soil suspension. The magnet, which originated from a mass spectrometer (MAT 150; Varian, Bremen, Germany), had a magnetic field strength of 5,000 G. The distance between the poles was 1.1 cm.

The suspension was transferred into the column and released with a flow rate of 100 ml min^{-1} by opening the regulation clamp at the bottom. This process was repeated twice without removing the column from the magnetic field. The magnetized particles collected at the points where the magnetic field lines intersected. The column was then removed from the magnetic field, and the remaining particles were suspended in 25 ml of washing buffer (0.15 M LiCl, 1 mM EDTA, 0.3% SDS-lithium dodecyl sulfate, 10 mM Tris-HCl [pH 7.5]). The column was again placed into the magnetic field, and the washing buffer was removed by opening the regulation clamp. This washing procedure was repeated until the washing solution became clear. This was usually the case after two washes. The column was then removed from the magnetic field, and the mRNA-hybridized Dynabeads were collected with 2 ml of 0.2 mM EDTA (pH 7.5) in a microtube. The tube was placed into a magnetic particle concentrator (MPC-E1/MPC-E, Dynal), and the EDTA solution was replaced by 50 μl of a fresh EDTA solution. The dissociation of the mRNA from the Dynabeads was performed by incubating the tubes for 10 min in a water bath at 68°C. To collect the Dynabeads, the tubes were again placed into the magnetic particle concentrator, and the mRNA solution was transferred into microtubes (0.5 ml) and stored at −20°C. The oligo(dT) Dynabeads could be reused after regeneration for at least three times. Regeneration of the remaining oligo(dT) Dynabeads was performed by resuspending the particles in 0.5 ml of 0.1 M NaOH for 2 min at 65°C in the water bath. After collection of the Dynabeads in the magnetic concentrator for 30 s, the supernatant was removed and discarded. This washing procedure was repeated until the pH of the washing solution was above 8.0, which was usually the case after three washing procedures. Finally, the Dynabeads were resuspended in 200 μl of storage buffer (250 mM Tris-HCl [pH 8.0], 20 mM EDTA, 0.1% Tween 20, 0.02% sodium azide).

Detection of aprotinin transcripts in mRNA extracted from soil. Aprotinin transcripts were detected with RT-PCR followed by a second PCR. For RT-PCR, amplifications of extracted soil mRNA, 2 μl, representing the amount extracted from 0.2 g of soil, were used as template. Both steps, RT and PCR, were performed successively in the same tube (0.5 ml). For the RT step, 0.75 μM each primer (Alf1 and Apr7), 4 μM deoxyribonucleoside triphosphates, 0.4 mM $MnCl_2$, 1× reverse transcriptase buffer, and *Tth* DNA polymerase (Boehringer) (4 U) were added to a final volume of 20 μl. The reverse transcriptase mixture was incubated for 20 min at 70°C. After incubation, the polymerase master mix (0.15 μM each primer, Alf1 and Apr7; 3.5 mM $MgCl_2$, 1× PCR buffer) was added to a final volume of 100 μl to each RT reaction, and 30 cycles were performed, each consisting of 1 min at 94°C, 1 min at 72°C, and 1 min at 50°C. To enhance the detection, 1 μl of the RT-PCR solution was used as a template in a second PCR. For this PCR, each tube contained, in a final volume of 50 μl, 0.5 μM each primer (Alf1 and Apr7), 500 μM $MgCl_2$, 0.8 μM deoxyribonucleoside triphosphates, 1× PCR buffer, and 4 U of *Taq* DNA polymerase (Boehringer).

PCR products were separated by horizontal agarose gel electrophoresis (1% agarose in Tris-borate-EDTA) and visualized by ethidium bromide staining and UV illumination (312 nm). To confirm the identity of PCR-generated products, Southern blot hybridizations with the DIG-labelled aprotinin gene probe were performed. The cDNA was transferred from agarose gels onto positively charged nylon membranes (Diagen, Hilden, Germany) with a vacuum filtration manifold (VacuBlot Transfer System, VAC-100; American Bionetics, Emeryville, Calif.). The membranes were hybridized with the indicated DIG-labelled probe, and hybridized products were detected as described for the colony hybridization.

Extraction of aprotinin from soil. Soil samples (5 g) were transferred to Oakridge centrifuge tubes (50 ml) and suspended in 5 ml of washing buffer (0.1 M Tris-HCl, 10% SDS, 5% [vol/vol] antifoam A [pH 7.0]) with a Vortex (2,500 rpm). Samples were then incubated in a shaking water bath (200 rpm; SW1; Julabo Labortechnik, Seelbach, Germany) at 40°C for 20 min. After centrifugation at 28,000 × *g* and 4°C for 10 min, the supernatant was transferred to prechilled Oakridge tubes. After 10 min of incubation in an ice bath, 5 ml of 3 M potassium acetate, pH 5.5, was added and carefully mixed by inversion and swirling on ice for another 10 min. The samples were then centrifuged at 28,000 × *g* and 4°C for 10 min, and the supernatant was transferred to prechilled Oakridge tubes. Precipitation of the aprotinin peptide was performed by adding 1 volume of ice-cold acetone. After 15 min of incubation on ice, the samples were centrifuged at 28,000 × *g* for 30 min and 4°C and the supernatant was discarded. The remaining pellet was dried for 10 min at room temperature, and the dried pellet was then dissolved in 150 μl of double-distilled water.

Detection and quantification of aprotinin extracted from soil. Aprotinin was detected in the soil-extracted fraction by an indirect ELISA, basically as described by Lam and Mutharia (21). An aprotinin-specific monoclonal antibody, purified with protein A (Pharmacia LKB GmbH, Freiburg, Germany), was a gift from Bayer AG. Standard humic acids used in inhibition assays were obtained from Aldrich-Chemie (Steinheim, Germany).

The soil-extracted aprotinin samples were pipetted in 50-μl portions (three replicates) into microtiter plate wells (MaxiSorb; oval bottom; Nunc, Wiesbaden, Germany). For adsorption of aprotinin, coating buffer (50 μl per well; 0.2 M sodium carbonate buffer, pH 9.6) was added. The microtiter plates were closed with a strip of parafilm (M; American National Can, Greenwich, Conn.) and incubated at 4°C for at least 16 h. The solution was removed. Each well was washed three times with 200 μl of washing buffer (0.05% Tween 20 in PBS [13.7 mM NaCl, 0.27 M KCl, 1.06 M Na_2HPO_4, 0.21 M KH_2PO_4]). Blocking reagent (200 μl per well; PBS with 1% skim milk [Difco]) was added, and the plates were incubated for 30 min at room temperature. The wells were washed again three times, each with 200 μl of washing buffer. The aprotinin antibody-containing solution (100 μl of a 1:250-diluted stock solution in PBS with 0.25% bovine serum albumin [BSA]) was added, and plates were incubated for 1 h at room temperature. Plates were washed three times as described above. The second antibody (anti-mouse immunoglobulin-DIG F(ab′) fragment [Boehringer Mannheim]), diluted 100-fold in PBS with 0.25% BSA, was added (100 μl per well). After 30 min of incubation at room temperature, the wells were washed as described above. The third antibody (anti-DIG-peroxidase, Fab fragments [Boehringer Mannheim]) was diluted in PBS with 0.25% BSA, 1:2,500-fold. Each well received 100 μl. After 30 min of incubation at room temperature, the solution was removed and the wells were washed three times as described above. Immediately after the last washing procedure, 100 μl of an ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt; Boehringer Mannheim) solution (1 mg of ABTS per ml in 0.05 M citrate buffer, pH 5.0) was added to each well; this was followed by the addition of the same volume of H_2O_2 (30% in 0.05 M citrate buffer, pH 5.0). After 10 min of incubation at room temperature, the A_{405} of each well was determined with a microtiter plate reader (vmax; Molecular Dynamics, Menlo Park, Calif.). Further readings were carried out after 30-min and 1-h incubations at room temperature.

RESULTS

Threshold for the detection of aprotinin gene transcripts in soil. Soil was inoculated with *S. cerevisiae* WHL292 or induced cells of *H. polymorpha* LR9-Apr4 at defined cell concentrations. With both strains, it was possible to detect the aprotinin transcripts from 10 cells g of $soil^{-1}$, corresponding to two cells per reaction tube in the RT-PCR procedure. To obtain visible products of the amplified mRNA on agarose gels, however, a second PCR had to follow the RT-PCR (Fig. 1A and Fig. 2). Without the second PCR, 10^6 cells g of $soil^{-1}$ were necessary to visually detect a product on agarose gels (data not shown). Transcripts could not be detected in unseeded soil or soil seeded with less than 10 cells g^{-1}. False-positive detection due to DNA contamination could be excluded because (i) no product occurred, when the RT step was omitted (Fig. 1A, lane 9;

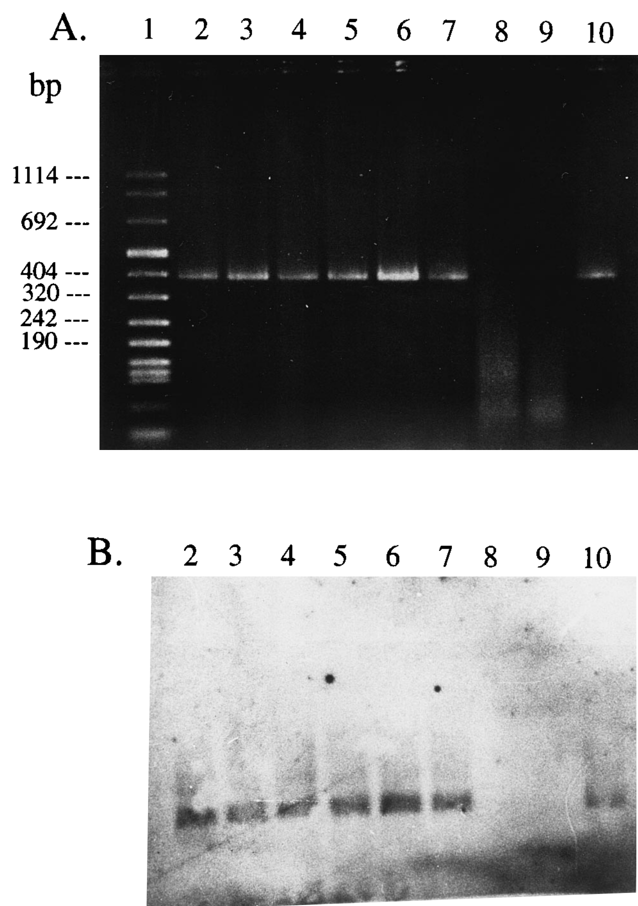

FIG. 1. (A) Gel electrophoresis of RT-PCR–PCR (RT-PCR followed by a second PCR; see Materials and Methods)-amplified products of mRNA extracted from soil seeded with *H. polymorpha* LR9-Apr4. (B) Southern hybridization of the products with the aprotinin gene probe. Lanes: 1, DNA size standards; 2 to 7, soil seeded with 10^6, 10^5, 10^4, 10^3, 10^2, and 10 cells per g, respectively; 8, control without methanol induction; 9, control without RT activity; 10, positive control with pure culture-extracted mRNA.

Fig. 2, lane 6) and (ii) only methanol-induced *H. polymorpha* cells generated a PCR product (Fig. 1A, lane 8). The identity of the amplified aprotinin gene sequence was confirmed with DNA-DNA hybridizations with an appropriate gene probe (Fig. 1B).

Aprotinin transcripts of the recombinant yeasts in soil. The fate of the aprotinin gene expression of *S. cerevisiae* WHL292 p707 was monitored in sterile soil, inoculated with 10^7 cells g^{-1}. The number of reisolated *S. cerevisiae* cells decreased by approximately 2 orders of magnitude within the 6-day-long experiment (Fig. 3A). Qualitative detection of transcripts was possible during the first 4 days but not after 6 days (Fig. 3B). Colony hybridization of *S. cerevisiae* WHL292 cells, reisolated after 6 days, with the aprotinin gene probe revealed that 1.4% (1.96×10^3 CFU g^{-1}) of the cultured cells still carried the aprotinin gene (data not shown). Therefore, it could be concluded that *S. cerevisiae* stopped its aprotinin gene expression after 6 days in soil.

Uninduced *H. polymorpha* LR9-Apr4 cells did not express the aprotinin gene in unamended soil (10^7 cells g^{-1}) (Fig. 4). The addition of methanol to inoculated soil samples did not cause toxicity when concentrations were below 5% methanol in the soil water (data not shown). When methanol was added to soil to yield a final concentration of 1% (vol/vol), no aprotinin gene expression could be detected. However, when methanol and a combined nutrient solution were added prior to the RNA extraction, aprotinin gene expression was observed (Fig. 4, lanes 4 and 8). Dilutions of the combined nutrient solutions did not cause gene expression. Thus, besides the inducer, methanol, sufficient nutrients were necessary to trigger the gene expression in soil.

In contrast to *S. cerevisiae* WHL292 p707, the survival and aprotinin gene expression of *H. polymorpha* LR9-Apr4 were studied in soil that was not presterilized. Cell numbers, as detected by colony hybridization, decreased from 10^6 CFU g of soil^{-1} to 1.4×10^4 within 6 days (data not shown). Induction of the aprotinin gene by prior application of the combined nutrient solution and methanol was possible throughout the experiment (Fig. 5).

Detection of the aprotinin peptide extracted from soil. Aprotinin could be specifically detected with an ELISA with monoclonal antiaprotinin mouse antibodies. The detection procedure involved two further, successively applied antibodies: first, an anti-mouse antibody which was DIG coupled, and, secondly, an anti-DIG antibody which was coupled with a peroxidase and therefore able to detect the antigen-antibody products with the colorimetric ABTS system. For aprotinin dissolved in water, the detection threshold was 50 pg of aprotinin per well, corresponding to 1 ng ml^{-1}. The threshold of detection for aprotinin extracted from sterile soil was 75 pg per well or, taking the amount of extracted soil into account, 45 pg g of soil^{-1} (Fig. 6).

Purified humic acids inhibited the detection of aprotinin by ELISA. The humic acid concentration required to reduce the detection of 50 pg of aprotinin by 50% was 5 μg ml^{-1}. In order to observe the same reduction with a 10-fold higher concentration of aprotinin, 70 μg of humic acids ml^{-1} was necessary (data not shown).

Occurrence and stability of aprotinin synthesized by the

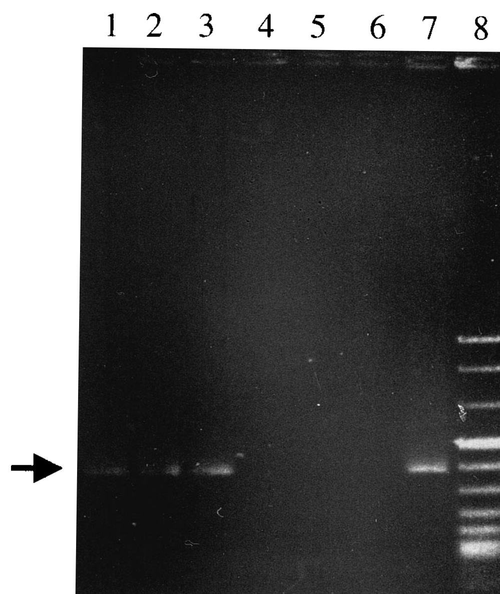

FIG. 2. Gel electrophoresis of RT-PCR–PCR-amplified products of mRNA extracted from *S. cerevisiae* WHL292 p707-seeded soil. Lanes: 1 to 4, 10^3, 10^2, 10, and 1 cell, respectively, per g of soil; 5, mRNA extracted from unseeded soil; 6, PCR without RT activity; 7, positive control with pure culture-extracted mRNA; 8, DNA size standards. Each lane represents 20% of the total PCR solution. The arrow indicates the position of the amplified aprotinin transcripts.

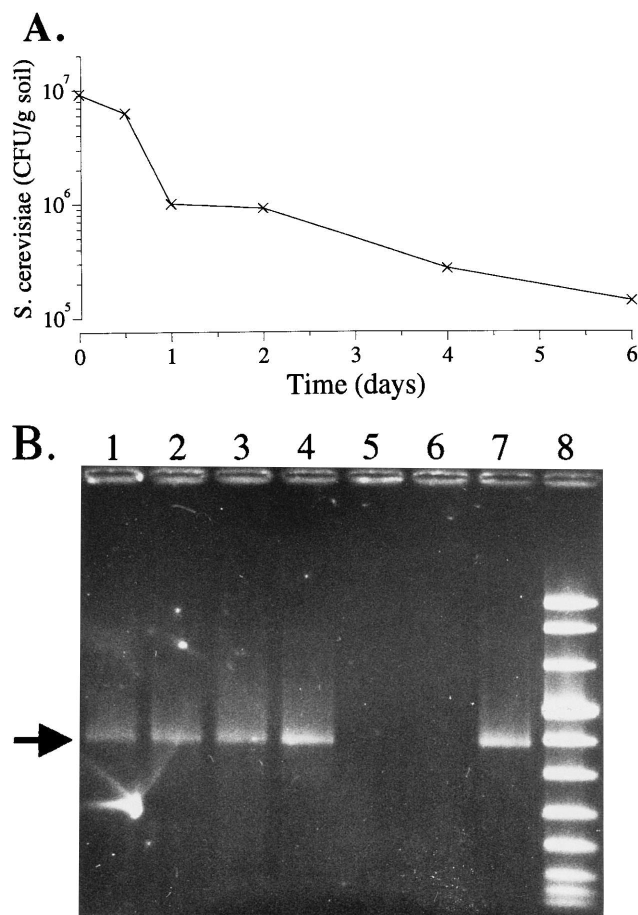

FIG. 3. Survival of *S. cerevisiae* WHL292 p707 inoculated into presterilized soil (A) and expression of the aprotinin gene (B). Extraction of mRNA followed by RT-PCR–PCR and detection of amplification products were performed after 0.5 (lane 1), 1.0 (lane 2), 2 (lane 3), 4 (lane 4), and 6 (lane 5) days of incubation. Other lanes: 6, negative control, no RT activity; 7, positive control with pure culture-extracted mRNA; 8, DNA size standards. The arrow indicates the position of the amplified aprotinin transcripts.

recombinant yeasts in soil. Pure cultures of *S. cerevisiae* WHL292 p707 produced in a batch fermentation 90 ng of aprotinin ml^{-1} when grown to stationary phase, and *H. polymorpha* LR9-Apr4 produced 64 ng ml^{-1} under the same conditions, but induced with 1% methanol (data not shown).

The secretion of aprotinin from yeast cells was monitored in presterilized and in nonsterile soil. In presterilized soil, the number of inoculated *S. cerevisiae* cells decreased by approximately 2 orders of magnitude within 2 weeks. In regular soil, the rate of decrease was slightly higher. Aprotinin accumulated in presterilized soil during the course of the experiment with decreasing production rates until day 10 (Fig. 7A). A total of 12 ng of aprotinin g of soil^{-1} was synthesized. In nonsterile soil, the initial production rates were similar to those of presterilized soil during the first 4 days. For 2 days, the aprotinin concentration remained constant and then decreased below the threshold of detection after 2 weeks (Fig. 7B). To observe the synthesis of aprotinin by *H. polymorpha* LR9-Apr4 in soil, the cells were induced as described for the mRNA extraction (3.5 h before extraction with 3.5% methanol solution in yeast medium). Compared with *S. cerevisiae* WHL292 p707, the initial aprotinin production rate of *H. polymorpha* LR9-Apr4 was twofold higher. The kinetics in soil not presterilized, however, were the same. An increase to 10 ng of aprotinin g of soil^{-1} within the first 4 days was followed by a decrease below the level of detection within 2 weeks (Fig. 7C).

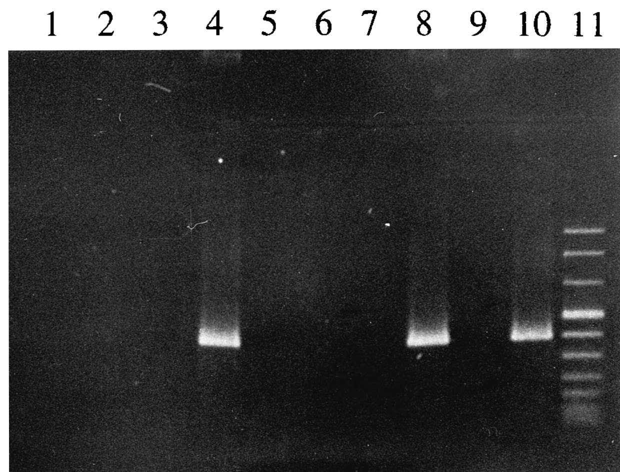

FIG. 4. Gel electrophoresis of RT-PCR–PCR-amplified products of aprotinin mRNA extracted from *H. polymorpha* LR9-Apr4-seeded, nonsterile soil. Initial cell concentration of *H. polymorpha* was 10^7 cells per g of soil. Soil samples were unamended (lane 1) or amended with combined nutrient solution (CNS) (for composition, see Materials and Methods) (lane 2), methanol (lane 3), CNS (10^{-1} dilution) and methanol (lane 4), CNS (10^{-2} dilution) and methanol (lane 5), CNS (10^{-3} dilution) and methanol (lane 6), CNS (10^{-4} dilution) and methanol (lane 7), or CNS (double concentrated) and methanol (lane 8). Other lanes include negative control without RT activity (lane 9), positive control (pure culture mRNA) (lane 10), and DNA size standards (lane 11).

DISCUSSION

Both yeast species investigated in this report do not belong to the common microflora of soils. They rather occur in habitats with higher concentrations of carbohydrates, such as flowers with nectar or fruit surfaces (6). In accordance with these ecophysiological profiles, we found that both *S. cerevisiae*

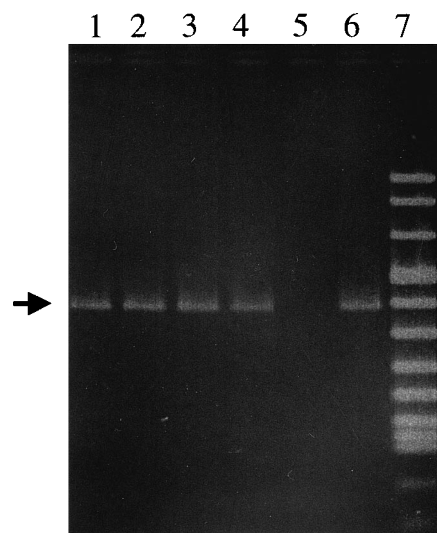

FIG. 5. Expression of the aprotinin gene by *H. polymorpha* LR9-Apr4 in nonsterile soil, detected after mRNA extraction, RT-PCR–PCR, and gel electrophoresis of the amplified product. Lanes: 1 to 4, 1, 2, 4, and 6 days of incubation, respectively; 5, negative control; 6, positive control; 7, DNA size standards. The arrow indicates the position of the amplified aprotinin transcripts.

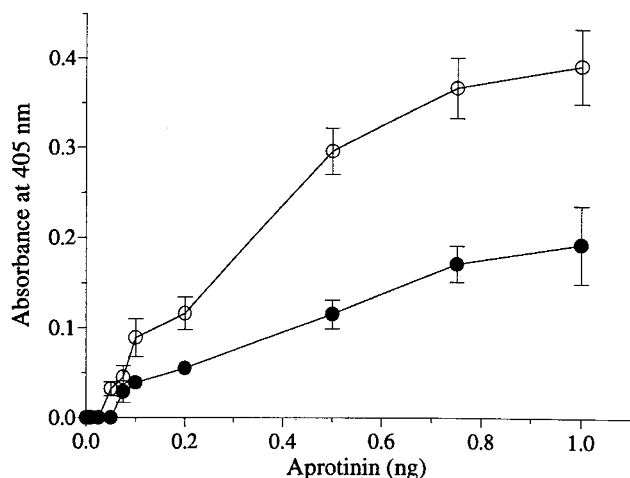

FIG. 6. Detection of aprotinin with ELISA. Results are shown for purified aprotinin dissolved in double-distilled water (○) and aprotinin extracted from soil (●).

WHL292 and *H. polymorpha* LR9-Apr4 were not able to persist in our bulk soil microcosms. Survival rates of *H. polymorpha* were higher than those for *S. cerevisiae*. Yeast populations, inoculated at 10^6 cells g of soil^{-1}, decreased within 2 weeks by 1 to 2 orders of magnitude with *H. polymorpha* and by 2 to 3 orders of magnitude with *S. cerevisiae*. The data are in accordance with survival rates of recombinant plasmid-bearing *S. cerevisiae* in soil suspensions as determined by Fujimura and coworkers (11). In contrast to these investigations, however, we observed a significant occurrence of plasmid segregants after 2 weeks in soil. The fact that both strains could not persist even in sterile soil (data not shown for *H. polymorpha*) indicated the inability to obtain nutrients from this environment (23). However, both species may also find microniches in the soil environment with higher chances for survival. Recently, several yeast species have been isolated from the gut of soil invertebrates (5).

To detect the aprotinin transcripts directly extracted from soil by RT-PCR, only two cells per PCR tube, corresponding to 10 cells per g of soil, were necessary. The detection threshold was similar to that of the aprotinin gene itself directly extracted from soil seeded with *S. cerevisiae* WHL292 p707, as shown in previous investigations (34). Compared with other methods developed for RNA extraction from soil (9, 36), no humic acids or other nucleic acids were coextracted because our isolation procedure extracted mRNA and not total RNA and utilized the magnetic field to separate the target sequences from other soil components.

H. polymorpha is a methylotrophic yeast. For heterologous gene expression, promoters regulating the key enzymes involved in the assimilation pathway of methanol, such as FMD, can be utilized (19, 22). Under pure culture conditions, the expression of the FMD promoter-controlled genes in *H. polymorpha* is induced by methanol and repressed by glucose. Additionally, in media which do not contain methanol but contain a nonrepressive carbon source such as glycerol, the enzyme can reach 30% of the induced level (13). Conditions in the nonamended soil environment did not induce FMD promoter-controlled gene expression. In contrast to pure culture, the addition of methanol was not sufficient to induce aprotinin synthesis. Transcription and aprotinin synthesis were observed only when a combined nutrient solution was concomitantly supplied with the inducer. We did not further identify the component of the combined nutrient solution which was re-

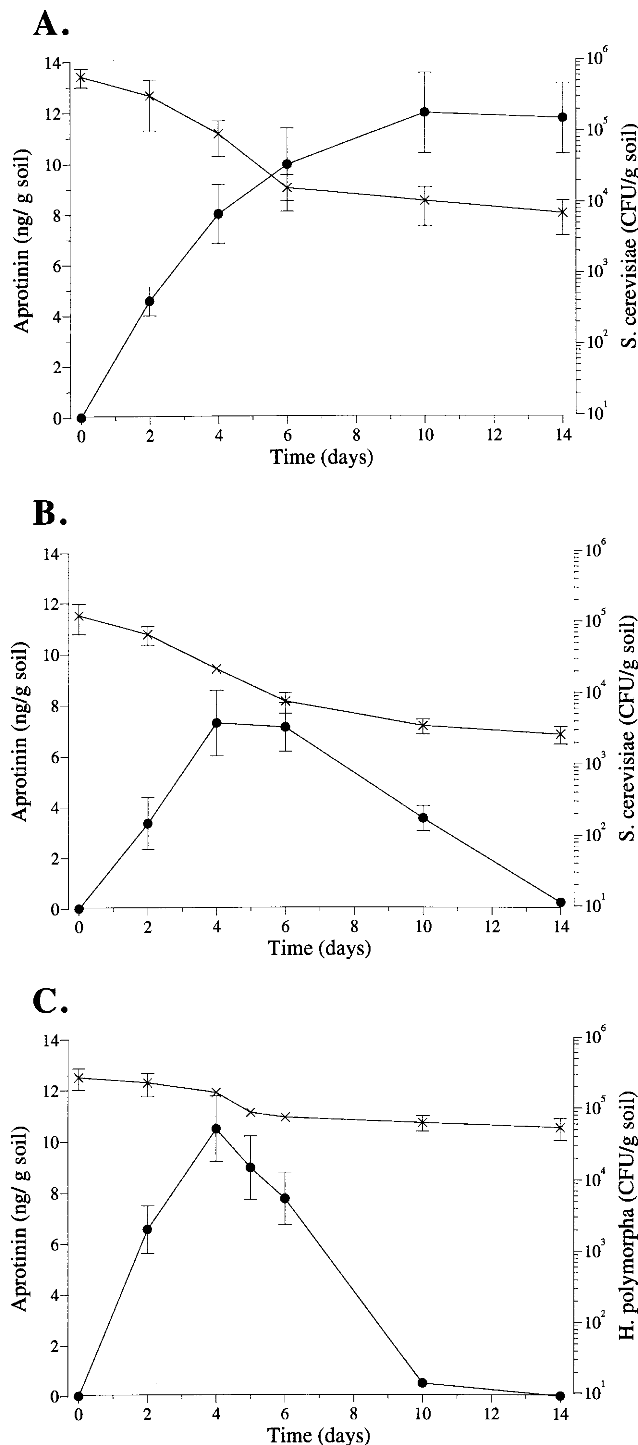

FIG. 7. Aprotinin production in sterile soil seeded with *S. cerevisiae* WHL292 p707 (A) and nonsterile soil seeded with *S. cerevisiae* WHL292 p707 (B) and *H. polymorpha* LR9-Apr4 (C). ●, aprotinin; ×, cell counts.

sponsible for gene expression. Since methanol itself can serve as both inducer and carbon source and the amount of 1% methanol (247 mM) in the soil water would have been sufficient to support growth, probably one or several components other than a carbon source were crucial for this gene expression. Generally, the use of promoters which are not inducible under conditions present in soil or aquatic environments (e.g.,

lack of inducer) can serve as a promising concept for the containment of recombinant gene products. On the other hand, even the constitutive expression of the aprotinin gene by *S. cerevisiae* in soil was reduced faster than indicated by the numbers of aprotinin gene-bearing cells. It is likely that the starvation of the yeast cells in soil resulted in a general reduction of gene expression activity. From naphthalene-contaminated soils, which were seeded with *Pseudomonas* strains, transcripts of the naphthalene catabolic genes were detectable only after addition of salicylate, a compound which can serve as both inducer and carbon source (28).

The parallel determination of transcription and translation allowed us to investigate the environmental impact on the two key processes in gene expression by our model organisms. Pichard and Paul (30) investigated the expression of a thermoregulated reporter gene of a marine *Vibrio* strain in the environment and found that the appearance of enzyme activity lagged 1 h behind the detection of mRNA. In experiments with sterile soil in our investigation, both detection methods yielded the same quality of information. The aprotinin gene was expressed during the first 4 days after inoculation, and significant aprotinin synthesis was observed during the same period of time. In nonpresterilized soil, however, the quality of information obtained with both methods was different. When soil was seeded with *H. polymorpha* and these cells were induced before extractions, transcript production could be detected for 6 days. However, because of the degradative activity of the indigenous microorganisms, the concentration of the gene product aprotinin already decreased after 4 days. The aprotinin formation rates in nonsterile and sterile soil were similar during the first 4 days. Probably, this was a result of an initial enrichment of aprotinin-degrading soil microorganisms.

The methods developed in this investigation can be used to study the expression of other genes from eukaryotic microorganisms directly in soil, if DNA (mRNA) sequence information or specific gene product antibodies are available. The mRNA extraction procedure described in this paper can potentially also be applied to the isolation of prokaryotic transcripts from soil, if the oligo(dT)-magnetic particle-poly(A) hybridization is replaced by other magnetic particles hybridizing directly or indirectly with transcript-specific sequences.

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REFERENCES

1. **Alard, P., O. Lantz, M. Sebagh, C. F. Calvo, D. Weill, G. Chavanel, A. Senik, and B. Charpentier.** 1993. A versatile ELISA-PCR assay for mRNA quantitation from a few cells. BioTechniques **15:**730–737.
2. **Bagdasarian, M., and M. M. Bagdasarian.** 1994. Gene cloning and expression, p. 406–417. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. ASM Press, Washington, D.C.
3. **Birnboim, H. C.** 1989. A rapid alkaline extraction method for the isolation of plasmid DNA, p. 175–187. *In* R. Wu, L. Grossman, and K. Moldave (ed.), Recombinant DNA methodology. Selected methods in enzymology. Academic Press, San Diego, Calif.
4. **Brake, A. J., J. P. Merryweather, D. G. Coit, U. A. Heberlein, F. R. Masiarz, G. T. Mullenbach, M. S. Urdea, P. Valenzuela, and P. J. Barr.** 1984. α-Factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **81:**4642–4646.
5. **Byzov, B. A., V. Ngugen Thanh, and I. P. Babjeva.** 1993. Yeasts associated with soil invertebrates. Biol. Fertil. Soils **16:**183–187.
6. **Campbell, R.** 1985. Plant microbiology. Edward Arnold (Publishers) Ltd, London.
7. **Cattaneo, R.** 1991. Different types of messenger RNA editing. Annu. Rev. Genet. **25:**71–88.
8. **Das, R. C., J. L. Shultz, and D. J. Lehman.** 1989. α-Factor leader sequence-directed transport of *Escherichia coli* β-galactosidase in the secretory pathway of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **218:**240–248.
9. **Fleming, J. T., J. Sanseverino, and G. S. Sayler.** 1993. Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. Environ. Sci. Technol. **27:**1068–1074.
10. **Fritz, H., and G. Wunderer.** 1983. Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. Arzneim.-Forsch./Drug Res. **33:**479–494.
11. **Fujimura, H., Y. Sakuma, and E. Amann.** 1994. Survival of genetically engineered and wild-type strains of the yeast *Saccharomyces cerevisiae* under simulated environmental conditions: a contribution on risk assessment. J. Appl. Bacteriol. **77:**689–693.
12. **Fuxa, J. R., G. W. Warren, and C. Kawanishi.** 1985. Comparison of bioassay and enzyme-linked immunosorbent assay for quantification of *Spodoptera frugiperda* nuclear polyhedrosis virus in soil. J. Invertebr. Pathol. **46:**133–138.
13. **Gellissen, G., Z. A. Janowicz, U. Weydemann, K. Melber, A. W. M. Strasser, and C. P. Hollenberg.** 1992. High-level expression of foreign genes in *Hansenula polymorpha*. Biotechnol. Adv. **10:**179–189.
14. **Gellissen, G., K. Melber, Z. A. Janowicz, U. M. Dahlems, U. Weydemann, M. Piontek, A. W. M. Strasser, and C. P. Hollenberg.** 1992. Heterologous protein production in yeast. Antonie Leeuwenhoek **62:**79–93.
15. **Goh, K. S., J. Hernandez, S. J. Powell, C. Garretson, J. Troiano, M. Ray, and C. D. Greene.** 1991. Enzyme immunoassay for the determination of atrazine residues in soil. Bull. Environ. Contam. Toxicol. **46:**30–36.
16. **Hall, J. C., L. K. Wilson, and R. A. Chapman.** 1992. An immunoassay for metachlor detection in river water and soil. J. Environ. Sci. Health B **27:**523–544.
17. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54:**703–711.
18. **Hutchinson, P. J., P. J. Shea, J. M. Takacs, and P. E. Staswick.** 1993. Immunoassay to detect enhanced carbamothioate degradation in soil. Weed Technol. **7:**396–403.
19. **Janowicz, Z. A., M. Eckart, C. Drewke, R. Roggenkamp, C. P. Hollenberg, J. Maat, A. M. Ledeboer, C. Visser, and C. T. Verrips.** 1985. Cloning and characterization of the *DAS* gene encoding the major methanol assimilatory enzyme for the methylotrophic yeast *Hansenula polymorpha*. Nucleic Acids Res. **13:**3043–3062.
20. **Jeffrey, W. H., S. Nazaret, and R. von Haven.** 1994. Improved method for recovery of mRNA from aquatic samples and its application to detection of *mer* expression. Appl. Environ. Microbiol. **60:**1814–1821.
21. **Lam, J. S., and L. M. Mutharia.** 1994. Antigen-antibody reactions, p. 104–132. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. ASM Press, Washington, D.C.
22. **Ledeboer, A. M., L. Edens, J. Maat, C. Visser, J. W. Bos, C. T. Verrips, Z. A. Janowicz, M. Eckart, R. Roggenkamp, and C. P. Hollenberg.** 1985. Molecular cloning and characterization of a gene coding for methanol oxidase in *Hansenula polymorpha*. Nucleic Acids Res. **13:**3063–3082.
23. **Liang, L. N., J. L. Sinclair, L. M. Mallory, and M. Alexander.** 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. Environ. Microbiol. **44:**708–714.
24. **Martensson, A. M., J. G. Gustafsson, and H. D. Ljunggren.** 1987. Competition in soil between effective and ineffective strains of *Rhizobium leguminosarum* biov. *trifolii* in the nodulation of red clover *Trifolium pratense* L. studied with ELISA. J. Gen. Microbiol. **133:**3467–3472.
25. **Nakayama, H., H. Yokoi, and J. Fujita.** 1992. Quantification of mRNA by non-radioactive RT-PCR and CCD imaging system. Nucleic Acids Res. **20:**4939.
26. **Nazaret, S., W. H. Jeffrey, E. Saouter, R. von Haven, and T. Barkay.** 1994. *merA* gene expression in aquatic environments measured by mRNA production and Hg(II) volatilization. Appl. Environ. Microbiol. **60:**4059–4065.
27. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates Inc., Sunderland, Mass.
28. **Ogunseitan, O. A., I. L. Delgado, Y.-L. Tsai, and B. H. Olson.** 1991. Effect of 2-hydroxybenzoate on the maintenance of naphthalene-degrading pseudomonads in seeded and unseeded soil. Appl. Environ. Microbiol. **57:**2873–2879.
29. **Pichard, S. L., and J. H. Paul.** 1991. Detection of gene expression in genetically engineered microorganisms and natural phytoplankton populations in the marine environment by mRNA analysis. Appl. Environ. Microbiol. **57:**1721–1727.
30. **Pichard, S. L., and J. H. Paul.** 1993. Gene expression per gene dose, a specific measure of gene expression in aquatic microorganisms. Appl. Environ. Microbiol. **59:**451–457.

31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **194:**3–21.
33. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54:**2185–2191.
34. **Tebbe, C. C., and W. Vahjen.** 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl. Environ. Microbiol. **59:**2657–2665.
35. **Tiedje, J. M., R. K. Colwell, Y. L. Grossman, R. E. Hodson, R. E. Lenski, R. N. Mack, and P. J. Regal.** 1989. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology **70:**298–315.
36. **Tsai, Y.-L., M. J. Park, and B. H. Olson.** 1991. Rapid method for direct extraction of mRNA from seeded soils. Appl. Environ. Microbiol. **57:**765–768.
37. **Tsuchiya, K., Y. Takahashi, K. Shohara, Y. Homma, and T. Suzui.** 1991. Rapid and simple ELISA procedure for the specific detection of *Pseudomonas cepacia* in soil. Ann. Phytopathol. Soc. Jpn. **57:**196–202.
38. **Voller, A., and D. E. Bidwell.** 1985. Enzyme immunoassays, p. 77–86. *In* W. P. Collins (ed.), Alternate immunoassays. John Wiley and Sons, Inc., New York.
39. **Waters, M. G., E. A. Evans, and G. Blobel.** 1988. Prepro α-factor has a cleavable signal sequence. J. Biol. Chem. **263:**6209–6214.