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# DNA Hybridization Probe for the *Pseudomonas fluorescens* Group

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**Plasmid pHF360 was constructed from cloned rRNA genes (rDNA) of *Pseudomonas aeruginosa* and used as hybridization probe for the *Pseudomonas fluorescens* group. The probe was tested by dot and in situ colony hybridizations to chromosomal DNAs from a wide variety of organisms. pHF360 DNA hybridized exclusively to chromosomal DNAs from bacteria representing the *P. fluorescens* group and separated them clearly from all other bacteria tested in the present study. Determination of the nucleotide sequence of the cloned DNA showed that it is a fragment from a 23S rRNA gene of *P. aeruginosa*. It was compared with the published 23S RNA sequence from *Escherichia coli*.**

The pseudomonads are a widely distributed group of bacteria. They show a great physiological versatility, and some of them play important roles as animal or plant pathogens. From 16S rRNA cataloging data (25) and from rRNA cistron homology studies (3, 17) it is known that *Pseudomonas* species represent a phylogenetically heterogeneous group of bacteria. The phylogenetic relationships of pseudomonads cover nearly the whole range of one main phylum of the eubacteria (26). On the other hand, there are many organisms not called pseudomonads that are closely related to some *Pseudomonas* species.

Until now the identification of *Pseudomonas* species required time-consuming biochemical tests (14, 18, 19). Recently a monoclonal antibody against an outer membrane protein from *Pseudomonas aeruginosa* has been described which is specific for both the *P. fluorescens* group and *Azotobacter* species (15). Two alternative approaches to a rapid identification of organisms or phylogenetic groups are in situ colony and dot hybridization techniques with specific hybridization probes. Randomly cloned chromosomal DNA fragments are used to differentiate *Bacteroides thetaiotaomicron* (21), or cloned genes or gene fragments serve as specific hybridization probes, e.g., the enterotoxin gene from *Vibrio cholerae* (11) or the rRNA genes from mycoplasmas (5, 20). The molecules of choice for phylogenetic studies are certainly the rRNAs. As known from 16S rRNA cataloging data and 16S rRNA and 23S rRNA total sequence data, the primary structure of these molecules is made up of regions of different conserved characters. In the present study we describe a DNA hybridization probe constructed from 23S rDNA of *P. aeruginosa* which is specific for representatives of the *P. fluorescens* group.

## MATERIALS AND METHODS

**Organisms and growth conditions.** The strains which we used are listed in Table 1. Pseudomonads were cultivated aerobically in 0.5% peptone–0.3% yeast extract broth (pH 7.0). *Azotobacter* and *Azomonas* species were cultivated in 0.01% $CaCl_2$–0.01% $MgSO_4 \cdot 7H_2O$–0.5% $Na_2MoO_4 \cdot 2H_2O$–0.09% $K_2HPO_4$–0.01% $KH_2PO_4$–0.001% $FeSO_4 \cdot 7H_2O$–0.5% $CaCO_3$–1.0% glucose broth (pH 7.3). Cells of *Chondromyces apiculatus*, *Spirulina maxima*, *Cytophaga* species, and *Flavobacterium* species were gifts from H. Reichenbach (Gesellschaft für Biotechnologische Forschung, Braunschweig, Federal Republic of Germany). *Methanococcus* cells were a gift from H. Märkl (Technische Universität München, Munich, Federal Republic of Germany). The other organisms were cultivated in 1.0% peptone–0.5% yeast extract–0.5% glucose–0.8% sodium chloride broth (pH 7.2). For cultivation on solid medium 1.3% agar was added.

**Isolation and labeling of rRNA and DNA.** Isolation and radioactive labeling (with $^{32}P$) of rRNA and DNA were carried out as previously described (23).

**Isolation and cloning of rDNA fragments.** Plasmid pHF1.1 (23), containing parts of 16S rRNA and 23S rRNA genes of *P. aeruginosa*, was treated with various restriction enzymes. The resulting fragments were separated by agarose gel electrophoresis (1.2% agarose–0.04 M Tris hydrochloride–0.02 M sodium acetate–0.002 M EDTA buffer, pH 7.9). After Southern hybridization (24) to 23S rRNAs of different strains, the chosen fragments were subcloned in vector pUN121 (16). Plasmids pHF1.1 and pUN121 were amplified in *Escherichia coli* RR28 (8).

**DNA sequencing.** For DNA sequencing the dideoxy method (22) and vector pUC8 (13) were used as described by Chen and Seeburg (2).

**Hybridizations.** Southern hybridizations of restriction fragments bound to nylon membrane (Zeta-Probe; Bio-Rad, Munich, Federal Republic of Germany) to $^{32}P$-labeled 23S rRNAs were carried out in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate, pH 7.0) containing 25 mM sodium phosphate buffer (pH 7.0), 2× Denhardt solution (0.04% polyvinylpyrrolidone, 0.04% Ficoll, 0.04% bovine serum albumin), 100 μg of yeast tRNA per ml, and 25% formamide at 50°C for 16 h. The conditions for rehybridization of the nylon membrane were those recommended by the manufacturer, with the exception that SSC and formamide were used in the concentrations mentioned above.

In situ colony hybridization was performed by the method of Grunstein and Hogness (7). For dot hybridization (10), 2 to 3 μg of chromosomal DNA, dissolved in 5 μl of bidistilled $H_2O$, was denatured at 95°C for 10 min, rapidly cooled to 0°C, and then spotted onto nitrocellulose filters (Schleicher & Schüll, Dassel, Federal Republic of Germany). DNA-rDNA hybridization experiments were performed in 3× SSC containing 25% formamide (optimal conditions), 35% formamide (stringent conditions), or 13% formamide (relaxed conditions) at 50°C for 18 h. Finally, the filters were washed once with 2× SSC containing 0.1% sodium dodecyl sulfate at

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TABLE 1. Strains studied, their phylogenetic positions, and reactions with pHF360 DNA probe

| Strain | No. and origin[a] | Phylogeny[b] | DNA-rRNA $T_{me}$[c] (°C) | Hybridization with pHF360 |
|---|---|---|---|---|
| *Pseudomonas aeruginosa* | DSM 50071$^T$ | PURC | 3.5 | + |
| *Pseudomonas fluorescens* | DSM 50090$^T$ | PURC | 0.0 | + |
| *Pseudomonas putida* | DSM 291$^T$ | PURC | 1.5 | + |
| *Pseudomonas aureofaciens* | DSM 50082 | PURC | 1.5 | + |
| *Pseudomonas syringae* | DSM 50252 | PURC | 2.0 | + |
| *Pseudomonas stutzeri* | DSM 50227 | PURC | 2.5 | + |
| *Pseudomonas mendocina* | DSM 50017$^T$ | PURC | 2.5 | + |
| *Pseudomonas chlororaphis* | DSM 50083$^T$ | PURC | 3.0 | + |
| *Pseudomonas pseudoalcaligenes* | DSM 50188$^T$ | PURC | 3.0 | + |
| *Pseudomonas alcaligenes* | DSM 50342$^T$ | PURC | 3.5 | + |
| *Pseudomonas cichorii* | DSM 50259$^T$ | PURC | 4.0 | + |
| *Azomonas agilis* | DSM 375$^T$ | PURC | 4.5 | − |
| *Azotobacter chroococcum* | DSM 2286$^T$ | PURC | 5.0 | − |
| *Alteromonas putrefaciens* | DSM 50427 | PURC | 12.0 | − |
| *Escherichia coli* | ATCC 11775 | PURC | 12.0 | − |
| *Pseudomonas maltophilia* | DSM 50170$^T$ | PURC | 13.0 | − |
| *Xanthomonas campestris* | DSM 1049 | PURC | 13.0 | − |
| *Proteus mirabilis* | DSM 788 | PURC | 13.5 | − |
| *Enterobacter aerogenes* | DSM 30053$^T$ | PURC | 14.0 | − |
| *Pseudomonas cepacia* | DSM 50181 | PURB | 15.5 | − |
| *Pseudomonas solanacearum* | NCP PB325 | PURB | 17.0 | − |
| *Pseudomonas delafieldii* | DSM 64$^T$ | PURB | 17.5 | − |
| *Pseudomonas palleroni* | DSM 63$^T$ | PURB | 17.5 | − |
| *Pseudomonas caryophylli* | DSM 50341$^T$ | PURB | 18.0 | − |
| *Pseudomonas acidovorans* | DSM 50251$^T$ | PURB | 19.0 | − |
| *Pseudomonas testosteroni* | DSM 38 | PURB | 19.0 | − |
| *Pseudomonas facilis* | DSM 649$^T$ | PURB | 20.0 | − |
| *Pseudomonas diminuta* | DSM 50635 | PURA | 19.0 | − |
| *Rhodopseudomonas capsulata* | DSM 1710$^T$ | PURA | 21.0 | − |
| *Chondromyces apiculatus* | DSM 436 | PURD | ND | − |
| *Staphylococcus aureus* | ATCC 12600$^T$ | Gm$^+$L | ND | − |
| *Staphylococcus capitis* | ATCC 27840$^T$ | Gm$^+$L | ND | − |
| *Bacillus pumilus* | DSM 27$^T$ | Gm$^+$L | ND | − |
| *Bacillus subtilis* | DSM 7 | Gm$^+$L | ND | − |
| *Bacillus cereus* | DSM 31$^T$ | Gm$^+$L | ND | − |
| *Bacillus alcalophilus* | DSM 485$^T$ | Gm$^+$L | ND | − |
| *Micrococcus luteus* | DSM 20030$^T$ | Gm$^+$L | ND | − |
| *Cytophaga lytica* | DSM 2039$^T$ | CFB | ND | − |
| *Flavobacterium breve* | ATCC 14234 | CFB | ND | − |
| *Spirulina maxima* | DSM B84.79 | CY | ND | − |
| *Herpetosiphon aurantiacus* | ATCC 23779$^T$ | CX | ND | − |
| "*Deinobacter* species"[d] | R 5004 | RD | ND | − |
| *Methanococcus vannielii* | DSM 1224$^T$ | ARC | ND | − |

[a] ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; R, Reichenbach, Gesellschaft für Biotechnologische Forschung, Braunschweig, Federal Republic of Germany.

[b] Abbreviations (26): Gm$^+$, gram-positive eubacteria; L, low G+C subdivision; H, high G+C subdivision; PUR, purple bacteria and relatives; A, B, C, and D alpha, beta, gamma, and delta (4a) subdivisions, respectively; CFB, flavobacteria and cytophagas; CY, cyanobacteria; CX, green nonsulfur bacteria and relatives; RD, radioresistant micrococci; ARC, archaebacteria.

[c] Melting point ($T_{me}$) differences between homologous and heterologous DNA-rRNA hybrids were taken from De Vos and De Ley (3). ND, Not determined.

[d] Radioresistant rods related to *Deinococcus* species (A. Reichenbach, W. Ludwig, and E. Stackebrandt, unpublished results).

50°C for 30 min, followed by two to four washings with 0.2× SSC– 0.1% sodium dodecyl sulfate for 30 min each at 50°C.

## RESULTS

**Screening and cloning of the rDNA probe.** Plasmid pHF1.1, containing the 5′ part of the 23S rDNA of *P. aeruginosa*, was restricted with *Hin*dIII. The fragments were analyzed by Southern hybridization with $^{32}$P-labeled 23S rRNAs from *P. aeruginosa*, *P. fluorescens*, *P. maltophilia*, *P. diminuta*, *P. acidovorans*, and *Micrococcus luteus* (Table 1). A 360-base-pair (bp) DNA fragment hybridized only with 23S rRNAs of *P. aeruginosa* and *P. fluorescens*, whereas no hybridization signals were obtained with 23S rRNAs from the less related *P. maltophilia*, *P. diminuta*, *P. acidovorans*, and *M. luteus*. These results indicate that the 360-bp fragment could possibly serve as a specific hybridization probe for the *P. fluorescens* group (3, 25). Therefore, the fragment was isolated by elution from agarose gel and cloned in the vector pUN121 for further analysis. The recombinant plasmid was designated pHF360.

**In situ and dot hybridizations.** The specificity of pHF360 was verified by in situ colony and dot hybridization to chromosomal DNAs from a variety of organisms (Table 1) by using $^{32}$P-labeled pHF360 as a probe. A clear separation could be obtained under optimal and stringent hybridization conditions. Stringent hybridization conditions resulted in a reduction of the intensity of the positive hybridization sig-


FIG. 1. Alignment of the sequence of the 360-bp fragment of 23S rDNA from *P. aeruginosa* (a) with the corresponding sequence of 23S rDNA of *E. coli* (b); identical bases in both sequences are indicated by asterisks.

nals, but the specificity did not change. Hybridization under relaxed conditions caused a loss of specificity, i.e., even DNAs of unrelated organisms often showed positive signals. These may result from nonspecific binding of pHF360 rDNA or vector-DNA to chromosomal sequences. Therefore we always used optimal conditions in our experiments. These conditions guaranteed reliable and strong hybridization signals for all *P. fluorescens* group DNAs and excluded hybridization with DNAs from other organisms. As positive and negative controls, chromosomal DNAs from *P. aeruginosa* (homologous DNA) and *Methanococcus vannielii*, representing the kingdom of archaebacteria, were applied. Colony hybridization was used for gram-negative bacteria, whereas dot hybridization was mainly used for gram-positive bacteria. After exposure for 20 to 48 h the autoradiograms showed positive hybridization signals only with DNAs from strains belonging to the *P. fluorescens* group. No positive signals could be detected with DNAs of any of the other gram-negative or gram-positive bacteria tested regardless of the method used.

Further colony hybridization experiments were carried out to prove the specificity of pHF360 with mixed cultures. Cells of *P. fluorescens* were added to cultures of the less closely related species *P. testosteroni* and *P. diminuta*. The percentage of *P. fluorescens* cells in the mixture varied from 2 to 30%. Monitoring the fluorescence at 360 nm allowed the differentiation of *P. fluorescens* colonies from those of *P. testosteroni* and *P. diminuta*. Only the *P. fluorescens* colonies hybridized with labeled pHF360 DNA (Fig. 1).

**Sequence analysis.** The nucleotide sequence of the 360-bp insert of pHF360 was determined by dideoxy sequencing (22) with universal sequencing and reverse sequencing primers (Boehringer GmbH, Mannheim, Federal Republic of Germany). Figure 2 shows the nucleotide sequence aligned to the corresponding part of the 23S rRNA from *E. coli* (1). The overall homology between the two sequences is about 78.8%. The localization of the corresponding region within a secondary structure model of a 23S rRNA gene is shown in Fig. 3.

## DISCUSSION

The differentiation of pseudomonads, especially in ecological or clinical studies, is difficult and time consuming. Therefore, we constructed a DNA hybridization probe specific for organisms of the *P. fluorescens* group. This probe allows the clear differentiation of *P. fluorescens* and its relatives from other pseudomonads and unrelated bacteria in a rapid hybridization assay. The hybridization probe contains a 360-bp fragment of the 23S rDNA from *P. aeruginosa*. *P. aeruginosa* is a representative of the *P. fluorescens* group. The 360-bp DNA fragment was cloned in the vector pUN121 and used as a probe in various hybridizations against chromosomal DNAs from a large number of organisms. The results showed that pHF360 is a specific hybridization probe for all members of the *P. fluorescens* group and separates them clearly from the closely related *Azotobacter* and *Azomonas* species and all other less closely related organisms.

The published total sequences of 23S rRNAs (1, 4, 6, 9, 12) are not yet sufficient to give indications about the absence or presence of convergent sequence homology in unrelated organisms. Therefore, we also hybridized pHF360 to chromosomal DNAs from representatives of the alpha, beta, and delta subgroups of the purple bacterial phylum (26), as well as to those of other eubacterial phyla and of the archaebacterial kingdom. No hybridization could be detected to these DNAs, indicating that no convergent homologies influenced the hybridizations.

Two different hybridization techniques were applied in our investigations: in situ colony hybridization and dot hybridization. Identical results were obtained with these two procedures. Many different DNAs can be analyzed simultaneously by dot hybridization. Definite amounts of pure DNA preparations can be spotted on filters, allowing comparable conditions for any tested sample. Gram-positive bacteria, whose cells are difficult to lyse, can only be investigated by dot hybridizations. The main disadvantage of the dot hybridization technique is the need to prepare DNA from each

ization technique is the need to prepare DNA from each organism. This time-consuming preparation is therefore the limiting step in this otherwise rapid technique. Thus, we are presently trying to devise methods for rapid preparation of DNA from gram-positive bacteria.

The in situ colony hybridization technique can be easily applied to gram-negative bacteria and is certainly faster than the dot hybridization. The great advantage of the in situ colony technique is the possibility of analyzing mixed cultures. Colony hybridization of *P. fluorescens*, *P. diminuta*, and *P. testosteroni* with pHF360 as a DNA probe gave good results when the cells were able to grow as single colonies. Only colonies of *P. fluorescens* reacted with the applied probe.

The 360-bp rDNA insert of pHF360 covers about 10% of the whole 23S rRNA gene. Sequence analysis of this fragment showed 78.8% nucleotide homology with the corresponding part of the 23S rRNA primary-structure of *E. coli*. Only a few short regions of higher homology are found in the 5'-terminal part of the fragment. These homologies apparently do not affect the specificity of the hybridization probe. *P. aeruginosa* and *E. coli* are both members of the gamma subgroup of the purple bacterial phylum but are only distantly related. Pure pHF360 DNA does not hybridize to *E. coli* chromosomal DNA. The pHF360 DNA probe, applied under standard hybridization conditions, allows rapid, reliable, and specific detection of pseudomonads belonging to the *P. fluorescens* group. A large number of organisms can be handled in one experiment. Since *P. fluorescens* and its relatives can be specifically detected in mixed cultures, the probe is also very useful for ecological and clinical studies. The recently described serological method using a monoclonal antibody against an outer membrane protein of *P. aeruginosa* exhibits a broader specificity than that using the pHF360 probe. It also reacts with the moderately related *Azotobacter* species group (15). Moreover, a point mutation may result in loss of the specific antigenic site, whereas single mutations should not affect the specificity of the 360-bp DNA probe. pHF360 together with other rDNA probes specific for *P. maltophilia*, *P. cepacia-P. acidovorans*, and *P. diminuta* should be sufficient to allow a rapid and simple differentiation of the heterogeneous group of pseudomonads.

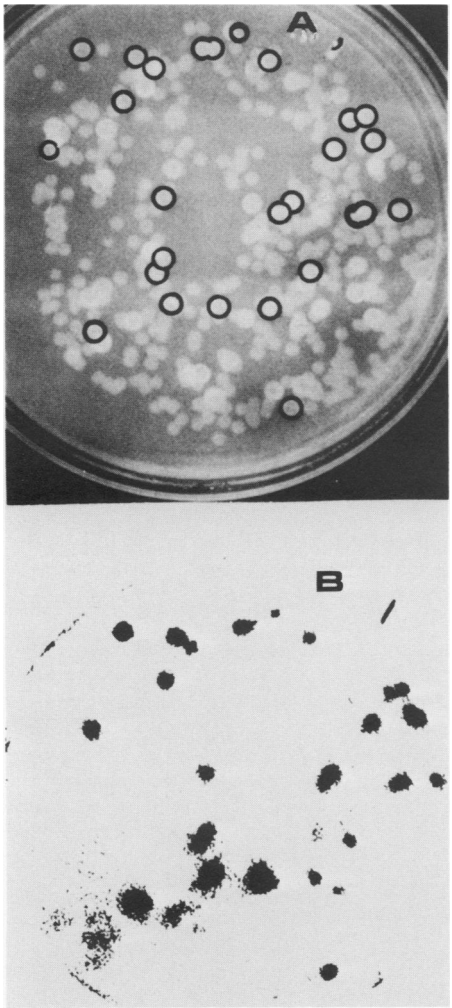


FIG. 2. Colony hybridization of a mixed culture to pHF 360. Cells of *P. fluorescens*, *P. diminuta*, and *P. testosteroni* were streaked on a nitrocellulose filter. (A) Aspect of the plate after overnight incubation. The fluorescing colonies of *P. fluorescens* are marked by black circles. (B) Autoradiogram of the hybridized filter. Only colonies of *P. fluorescens* gave a positive signal.

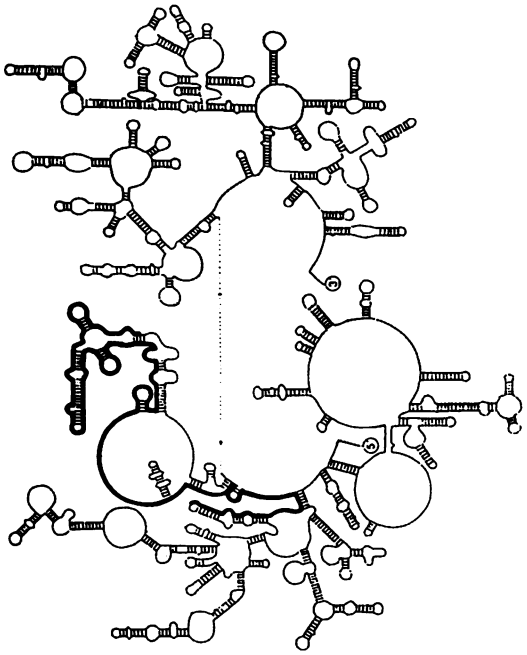

FIG. 3. Secondary structure model of a 23S rRNA gene from Douglas and Doolittle (4); the thick line indicates the localization of a cloned 23S rRNA gene fragment (insert of pHF360) of *P. aeruginosa* DSM 50071.

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