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Development of Gene-Probes for *Azospirillum* Based on 23S-rRNA Sequences

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Abstract

Highly variable stretches of the 23S ribosomal RNA molecule of the four *Azospirillum* species were amplified via polymerase chain reaction (PCR) and the cloned products were sequenced. The primary structures of the rDNA were analyzed and oligonucleotide probes were designed, in order to target to the 23S-rRNA of *Azospirillum* ssp. with a specificity at the genus and species level. The selective binding of the phylogenetic probes to immobilized nucleic acids of *Azospirillum* ssp. could be demonstrated in dot blot and colony hybridization experiments.

Keywords: Polymerase Chain Reaction (PCR), sequencing, hybridization, gene probe, oligonucleotide, 23S-ribosomal RNA

1. Introduction

A specific detection method for individual bacterial strains or groups of bacteria is a prerequisite for the population analysis of autochtonous soil microbes and the monitoring of microbes released in the environment. Traditional methods are limited, because they require cultivation and they are mostly non-discriminatory. New molecular techniques are now available to overcome these limitations. One possibility thereby is the probing of immobilized DNA fixed

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to membranes in hybridization experiments with phylogenetic probes. The advantages of this strategy are the high sensitivity and practicability of performance and the applicability not only upon cultivation of microbes but also with nucleic acid from environmental samples directly. Beyond this, pylogenetic gene probes with specificities from species up to kingdom level can be constructed. Probes derived from ribosomal RNA (rRNA) sequences are sensitive and specific because of the high copy number (10^5 per cell) of target molecules in a bacterial cell and the presence of unique and conserved regions in rRNA (Woese, 1987). 16S-rRNA and 23S-rRNA sequences are ideal molecules to design gene probes because they contain both conserved and variable segments (Pace et al., 1986). Probes based on 16S-rRNA sequences are more common, but the 23S-rRNA molecule offers more possibilities because of its larger information content (higher length: about 3000 basepairs, more variations in secondary structure) (Höpfl et al., 1989).

In our approach, *Azospirillum* ssp. were used as a model organism to design specific gene probes. These rhizosphere bacteria, which have a plant growth promoting capacity, are interesting candidates for agronomic application. Species-specific gene probes can be used for a taxonomic evaluation of isolates on a phylogenetic basis and could be helpful to get insight into the population dynamics of these bacteria in natural habitats.

2. Materials and Methods

Bacterial strains and plasmids are listed in Table 1.

Media and growth conditions

Azospirillum strains were grown either in mineral medium or in rich medium (NB, Merck, Darmstadt), after adding buffer and salt solutions (Hartmann et al., 1988a; Hartmann, 1988b). The *E. coli* strains were cultivated in TY-medium (10 g Tryptone, 5 g yeast extract, 5 g NaCl, ad 1 l aq. dem., pH 7.2). The growth temperature for the *Azospirillum* ssp. was 33°C, except for *A. halopraeferens* it was 41°C. The *E. coli* strains were incubated at 37°C. For recombinant *E. coli* strains ampicillin (100 mg/l) and x-Gal (4-chlor-3-indolyl-b-D-galactopyranoside; 40 mg/l) were added to select for plasmids carrying cloned DNA.

Table 1. Bacterial strains and plasmids

Bacteria:

Name	Strain	Reference
Azospirillum amazonense	DSM 2787=Y1	Magalhaes et al., 1983
Azospirillum brasilense	DSM 1690=Sp7	Tarrand et al., 1978
Azospirillum halopraeferens	DSM 3675=Au4	Reinhold et al., 1987
Azospirillum irakense	CIP 10311=KBC1	Khammas et al., 1989
Azospirillum lipoferum	DSM 1691=Sp59b	Tarrand et al., 1978
Escherichia coli	DSM 3947	Vieira and Messing, 1982
Escherichia coli	DSM 498	K12 "wildtype"

Plasmids:

Name	Host strains	Reference
pBluescript KS+	JM83 and derivatives	Stratagene

DSM= Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG
CIP= Collection de l'Institut Pasteur, Paris, France

Nucleic acid isolation and analysis

The bulk nucleic acid (NA) of each strain was prepared following the rapid isolation method of Stahl and Flesher (1987). Bulk NA was used in a standard polymerase chain reaction (PCR; Saiki et al., 1988) to amplify parts of the 23S-rDNA. After purification with glasmilk^{TM} (Dianova, Hamburg) a restriction enzyme digest was performed. The DNA was ligated to the appropriate restricted vector pBS KS+. Transformation of competent *E. coli* JM 83 cells was carried out according to Cohen et al. (1972) and Cohen et al. (1973). Plasmid DNA was isolated from agardish cultures using the rapid boiling preparation method (Wang et al., 1988). Positively screened recombinant plasmids were sequenced using the didesoxynucleotide chain-termination method (Sanger et al., 1977).

Oligonucleotide synthesis and labelling with ^{32}P

The synthesis of the oligonucleotides were performed with the gene assembler Plus (Pharmacia/LKB, Freiburg), using the solid-phase phosphoamidite method (Mateucci and Caruthers, 1981) and purified by gel filtration (NAP 5-Columns, Pharmacia, Freiburg). The oligonucleotide probes were labelled with γ-^{32}P-ATP by T4-polynucleotide kinase reaction (Sambrook et al. 1989).

Dot blot- and colony-hybridization

For dot blot hybridization (Kafatos et al., 1979) the crude NA was fixed on Zeta-probe nylon filters (BioRad, München) by a dot blot apparatus (BRL, Eggenstein) and immobilized by drying overnight. Prewashing (solution: 0.1×SSC and 0.5% SDS; 1 hr) and prehybridization treatment (solution: 5×SSC, 5×Denhard solution and 1% Sarkosyl; 1 hr) was done at the appropriate temperature (Suggs et al., 1981). Hybridizations with specific and universal probes in the prehybridization solution were performed subsequently for 4–24 hrs. The filters were washed twice in 2×SSC, 0.1% SDS at room temperature for 20 min and once at hybridization temperature for 5 min. The signals were detected by autoradiography. Stripping off the probe was carried out (80°C; solution: 0.1×SSC and 0.5% SDS) until no more radioactivity was detectable.

Immobilization of colonies and nucleic acid disintegration was performed using a method described by Betzl et al., 1990. The hybridization solutions were performed following the description of the membrane manufacturer. The hybridization procedure and signal detection resembled the dot blot hybridization.

3. Results and Discussion

Design of species and genus specific probes

One striking feature of 23S-rRNA primary structure is the presence of a stretch located in helix 56–59, known to be highly variable in base composition also at species level (Höpfl et al., 1989). For the construction of the species- and genus-specific gene probes, sequences containing this region were determined for all four *Azospirillum* species. This was done by amplification of 23S-rDNA stretches via PCR, ligation with the high-copy vector pBS KS+, cloning in *E. coli* JM83 and subsequent enzymatic sequencing.

The sequenced parts of the 23S-rDNA of all four *Azospirillum* ssp. were analyzed by computer. The sequences were compared to corresponding parts of 23S-rDNA sequences of a broad spectrum of other microorganisms (Ludwig, personal communication). Stretches of 15 to 17 nucleotides length were traced out to be suitable for species- or genus-specific probes. The sequences of the stretches selected as target sequences and the dissociation temperatures (T_D) of the complementary synthesized oligonucleotide (DNA-rRNA-hetero-duplex) are shown in Table 2. The T_D were calculated according to the base composition and length of the oligonucleotide using the formula of Suggs et al. (1981): $T_D = 4(G+C) + 2(A+T)$ [C°]. The temperature chosen for hybridization lies 5 K below this value.

Table 2. 23S-rRNA-targeting sequences for probe design

Name	Sequence	T_D
AA-Oligo	5′-GTG TGC CAT GGA GGT GT-3′	54°C
AB-Oligo	5′-GCC CGG CTG GGG ACC C-3′	60°C
AH-Oligo	5′-AGC GTG CTG CGG CGA-3′	52°C
AL-Oligo	5′-TAG CCC CGC CTT ATA-3′	46°C
AZO-Oligo	5′-CCC WGG AAA YAG CCC C-3′	52/53°C
UNIV-Oligo	5′-AAA CCG ACA CAG G-3′	40°C

AA: *Azospirillum amazonense*, **AB:** *Azospirillum brasilense*; **AH:** *Azospirillum halopraeferens*; **AL:** *Azospirillum lipoferum*; **AZO:** genus *Azospirillum*; **UNIV:** universal probe, targeting a highly conserved region of the 23S-rRNA, positioned at the stretch basepair No. 1592 to 1606 corresponding to the *E. coli* 23S-rRNA numeration; $\mathbf{T_D}$: temperature of dissociation

Oligonucleotides were synthesized following the method of Mateucci and Caruthers (1981). The advantage of the hybridization technique using oligonucleotides is the high sensitivity. A single basepair mismatch drastically affects the stringency of the heteroduplex.

Hybridization experiments

The availability of target rRNA of the four *Azospirillum* ssp. and *E. coli* K12 as reference was confirmed by dot blot hybridization to an oligonucleotide probe directed to a highly conserved stretch of the 23S-rRNA (see Fig. 1C). Hybridization to the genus specific probe resulted in signals at the positions of the *Azospirillum* NA dots (see Fig. 1B). Nucleic acid of *E. coli* K 12 did not bind detectable amounts of this probe.

The specificity of the species-specific probes was tested in four distinct hybridization experiments (see Fig. 1A). Under stringent (5 K below T_D) or more relaxed (15 K below T_D) conditions the same pattern of positive signals were obtained. In each case only the corresponding rRNA bound the species-specific probe significantly. The same results were obtained including the nucleic acid of *Azospirillum irakense* immobilized on the membrane. Only the oligonucleotide probe for the genus *Azospirillum* was bound by the immobilized nucleic acid of *Azospirillum irakense.*

During an inoculation experiment of wheat roots with *Azospirillum brasilense* Sp 7, the probe "AB-Oligo" was used in a colony-hybridization experiment. The successful identification of *Azospirillum brasilense* colonies among 100 times background colonies was possible (data not shown).

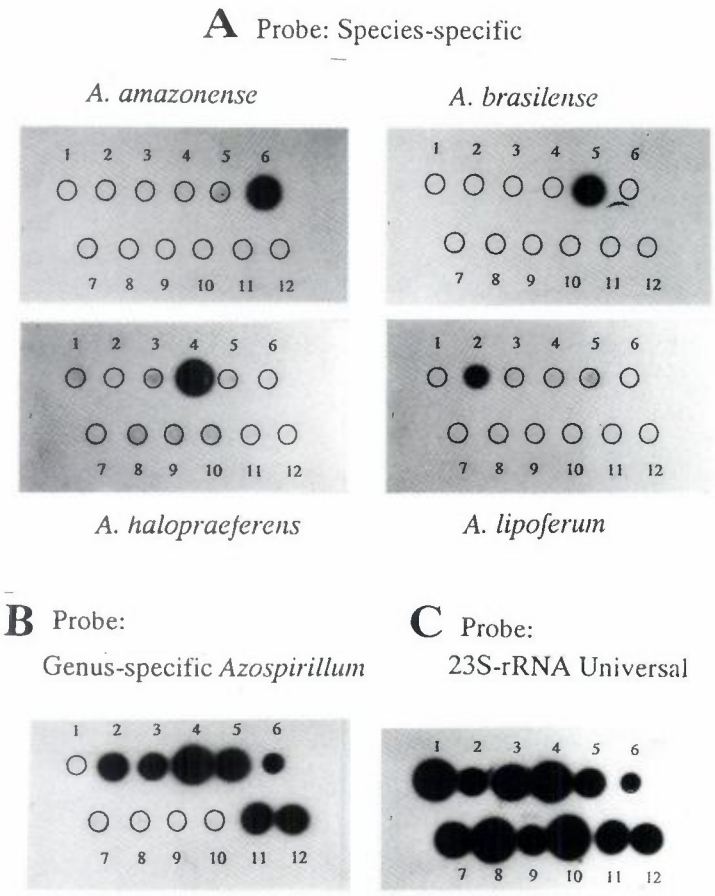

Figure 1. Testing the specificity of 23S rRNA derived *Azospirillum* gene probe by dot blot hybridization.
(A) Hybridization with species-specific gene probes.
(B) Hybridization with the genus-specific gene probe.
(C) Proof of availability of rRNA target sequences using a universal 23S-rRNA oligonucleotide probe directed against a highly conserved sequence of this molecule.

Dot pattern of each filter: **row A**: 1&2, *A. amazonense* (5 and 0.5 μg NA); 3&4, *A. brasilense* (5 and 0.5 μg NA); 5, *E. coli* (5 μg NA)
row B: 1&2, *A. halopraeferens* (5 and 0.5 μg NA); 3&4, *A. lipoferum* (5 and 0.5 μg NA); 5, *E. coli* (0.5 μg NA).

4. Conclusions and Further Prospects

The results indicate, that the designed probes targeting the rRNA of *Azospirillum* ssp. could be a potent tool for *Azospirillum* taxonomy and further investigations of *Azospirillum* ecology. The specificity has to be improved by screening with a broad spectrum of bacteria, especially closely related organisms. In further investigations, it could be possible to attach these probes to fluorochromes, enabling the detection of single cells by *in situ* hybridization (Amann et al., 1990a) or the use in flow cytometry for quantification analysis (Amann et al., 1990b). The combination of the *Azospirillum* probes with RFLP-analysis and pulsed field gel electrophoresis (PFGE) could improve the applicability of this technique (Gündisch et al., 1991).

The comparative monitoring with strain specific monoclonal antibodies (Schloter et al., 1992) and with gene probes may gain more fundamental insight into the fate of inoculants and to complex community behaviour and ecological processes of *Azospirillum* in the rhizospere at different environmental conditions.

REFERENCES

Amann, R.I., Krumholz, L., and Stahl, D.A. 1990a. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **177:** 762–770.

Amann, R.I., Binder, B.J., Olsen, R.J., Chrisholm, S.W., Devereux, R., and Stahl, D.A. 1990b. Combination of 16S-rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56:** 1919–1925.

Betzl, D., Ludwig, W., and Schleifer, K.-H. 1990. Identification of *Lactococci* and *Enterococci* by colony hybridization with 23S-rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **56:** 2927–2929.

Cohen, S.N., Chang, A.C.Y., Boyer, H.W., and Helling, R.B. 1973. Construction of biologically functional bacterial plasmid *in vitro*. *Proc. Natl. Acad. Sci. USA* **70:** 3240–3244.

Cohen, S., Chang, A., and Hsu, L. 1972. Non chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69:** 2110–2114.

Gündisch, C., Bode, W., Kirchhof, G., and Hartmann, A. 1991. Characterization of *Azospirillum* strains by endonuclease restriction length polymorphism using pulsed field gel electrophoresis. *5th International Workshop on* Azospirillum, Wennigsen/Deister, F.R.G., Sept. 4–7 1991, book of abstracts.

Hartmann, A., Fu, H., and Burris, R.H. 1988a. Influence of amino acis on nitrogen fixation ability and growth of *Azospirillum* ssp. *Appl. Env. Microbiol.* **54:** 87–93.

Hartmann, A. 1988b. Osmoregulatory properties of *Azospirillum* ssp.. In: Azospirillum *IV-Genetics, Physiology, Ecology.* W. Klingmüller, ed. Springer-Verlag, Berlin, pp. 122–130.

Höpfl, P., Ludwig, W., Schleifer, K.-H., and Larsen, N. 1989. The 23S ribosomal RNA higher-order structure of *Pseudomonas cepacia* and other prokaryotes. *Eur. J. Biochem.* **185:** 355–364.

Kafatos, F.G., Jones, C.W., and Efstradiadis, A. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridisation procedure. *Nucl. Acids. Res.* **7:** 1541–1552.

Khammas, K.M., Ageron, E., Grimont, P.A.D., and Kaiser, P. 1989. *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soil. *Res. Microbiol.* **140:** 679–693.

Mateucci, M.D. and Caruthers, M.H. 1981. Synthesis of deoxyoligonucleotides on a polymer support. *J. Am. Chem. Soc.* **103:** 3185–3191.

Magalhaes, F.M., Baldani, J.I., Souto, S.M., Kuykendall, J.R., and Döbereiner, J. 1983. A new acid-tolerant *Azospirillum* species. *An. Acad. Brasil. Scienc.* **55:** 417–430.

Pace, N.R., Stahl, D., Lane, D., and Olsen, G. 1986. The analysis of natural microbial populations by ribosomal RNA sequences. *Adv. Microbial Ecol.* **9:** 1–56.

Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, M., Kersters, K., Thielemans, S., and De Ley, J. 1987. *Azospirillum halopraeferens* sp. nov., a nitrogen fixing organism with roots of Kallar grass (*Leptochloa fusca* (L.)) Kunth). *Int. J. Syst. Bacteriol.* **37:** 43–51.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **293:** 487–491.

Sambrook, J., Fritsch, E., and Maniatis, T. 1989. *Molecular Cloning-A Laboratory Manual.* 2nd ed. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, Chapter 11.31.

Sanger, F., Nicklen, S., and Coulson, A. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463-5467.

Schloter, M., Bode, W., Hartmann, A., and Beese, F. 1992. Sensitive chemoluminescence-based immunological quantification of bacteria in soil extracts with monoclonal antibodies. *Soil Biol. Biochem.* (in press).

Stahl, D.A. and Flesher, B. 1987. *Applications of ribosomal RNA sequencing to studies of microbial ecology.* Gray Freshwater Biological Institute, Summer Course July 6–10.

Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K., and Wallace, R.B. 1981. Use of synthetic oligodeoxynucleotide for the isolation of specific cloned DNA sequences. In: *Developmental Biology Using Purified Gases.* D. Braun and C.F. Fox, eds. Academic Press, New York, pp. 683–693.

Tarrand, J.J., Krieg, N.R., and Döbereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with description of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can. J. Microbiol.* **24:** 967–980.

Vieira, J. and Messing, J. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19:** 259–268.

Wallace, R.B., Shaffer, J., Murphey, R.F., Bonner, J., Hirose, T., and Itakura, K. 1979. Hybridization of synthetic oligodeoxyribonucleotides to FX 174: the effect of single base pair mismatch. *Nucl. Acid Res.* **6:** 3543–3557.

Wang, L.M., Weber, D.K., Johnson, T., and Sakaguchi, A.Y. 1988. Supercoil sequencing using unpurified temperature produced by rapid boiling. *BioTechniques* **6:** 839–843.

Woese, C.R. 1987. Bacterial Evolution. *Microbiol. Rev.* **51:** 221–271.