

## Design and Application of New 16S rRNA-targeted Oligonucleotide Probes for the *Azospirillum-Skermanella-Rhodocista*-Cluster

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### Abstract

The genera *Azospirillum*, *Skermanella* and *Rhodocista* form a phylogenetic subgroup within the alfa subclass of *Proteobacteria*. Based on comparative 16S rRNA sequence analysis a nested set of new oligonucleotide probes was designed. It comprises probes for the whole genus cluster *Azospirillum-Skermanella-Rhodocista*, for the *Azospirilli* subcluster I including *A. lipoferum*, *A. doebereineriae*, *A. largimobile*, *A. brasilense* and *A. halopraeferens*, for the *Azospirilli* subcluster II including *A. amazonense*, *A. irakense* and the genus *Skermanella*, for the genus *Rhodocista* as well as for all *Azospirilli* species or species cluster. The new probes allow a fast and reliable *in situ* identification of bacteria belonging to the *Azospirillum-Skermanella-Rhodocista*-cluster at different phylogenetic levels. The specificity of the new probes was tested with 56 strains of the *Azospirillum-Rhodocista-Skermanella*-cluster and selected reference cells from other genera by hybridising with the complete probe set. In addition, applications of the fluorescently labelled probes for *in situ* identification of isolates and for the *in situ* localisation of *A. brasilense* on maize roots were demonstrated using confocal laser scanning microscopy.

**Key words:** *Azospirillum* – *Rhodocista* – *Skermanella* – fluorescence *in situ* hybridization – FISH – 16S rRNA – top to bottom approach – identification – monitoring

### Introduction

Bacteria belonging to the genus *Azospirillum* are frequently studied nitrogen-fixing soil bacteria. *Azospirilli* have a worldwide distribution and can be isolated from tropical, subtropical and temperate zones of the world. They occur in association with roots, stems and leaves of a large variety of gramineous and non-gramineous plants, e.g. numerous wild and cultivated grasses and cereals, legumes, vegetables and fruit trees (BASHAN and HOLGUIN, 1997; GUNARTO et al., 1999, HAN and NEW, 1998, WEBER et al., 1999).

*Azospirilli* are in the centre of scientific interest for the last two decades because under appropriate conditions members of this genus can enhance plant development and promote the yield of several agriculturally important crop plants. The mechanisms by which plant growth promoting rhizobacteria (PGPR) support the growth of plants have not been elucidated completely, but several possible modes of

action have been proposed: (1) stimulation of the growth of the entire root system which results in enhanced mineral and water uptake (SAUBIDET and BARNEIX, 1998), (2) biological nitrogen fixation which directly contribute nitrogen to the plant, (3) bacterial production and release of phytohormones including auxins, gibberellins and cytokinins (OKON and VANDERLEYDEN, 1997) that play an essential role in plant growth stimulation in general (BASHAN and HOLGUIN, 1997), (4) bacterial nitrite production which increase the formation of lateral roots (BOTHE et al., 1992), (5) displacement of plant pathogens i. e. fungi and bacteria by antimicrobial activity (RODELAS et al., 1999; SHAH et al., 1992; BASHAN and HOLGUIN, 1997). The application of *Azospirillum* inoculants as a “green fertilizer” has been carried out with considerable success (OKON and LABANDERA-GONZALEZ, 1994).

Besides the development of commercial *Azospirillum* inoculants, Azospirilli are also under discussion for the industrial production of poly- $\beta$ -hydroxybutyrate for medical use (OKON and ITZIGSOHN, 1992), production of vitamins (RODELAS et al., 1993; DAHM et al., 1993), for the degradation of pollutants (RUSSEL and MUSZYNSKI, 1995), purification of urban residual water and the breakdown of cellulose in combination with cellulolytic bacteria (BASHAN and HOLGUIN, 1997).

Bacteria of the genus *Azospirillum* are Gram-negative, nitrogen fixing rods and comprise of 7 species: *A. lipoferum*, *A. irakense*, *A. halopraeferens*, *A. brasilense*, *A. amazonense*, *A. largimobile* (formerly *A. largomobile* and *Conglomeromonas largomobilis* subsp. *largomobilis*) and the recently described *A. doebereineriae* (ECKERT et al., 2001). Together with the nitrogen-fixing non sulfur, purple phototrophic bacteria *Rhodocista centenaria* and the non nitrogen-fixing *Skermanella parooensis* (formerly *Conglomeromonas largomobilis* susp. *parooensis*) the genus *Azospirillum* forms a phylogenetic subgroup within the alfa-subclass of *Proteobacteria* (Azospirilli subcluster).

For both, the agriculturally and industrial applications of *Azospirillum* reliable and rapid identification as well as monitoring tools are necessary. Several methods for identification have been reported. These include traditional physiological methods (DOEBEREINER, 1995) and serological and molecular determinations (KIRCHHOF et al., 1997). Most recently, FANCELLI et al. (1998) described the development of randomly amplified polymorphic DNA markers for the detection of *Azospirillum* strains in soil. Different techniques are available for direct *in situ* identification and localisation of Azospirilli on inoculated roots. ASSMUS et al. (1997) used fluorescently labelled oligonucleotide probes and antibodies in combination with confocal scanning laser microscopy for the *in situ* localisation of *A. brasilense* on wheat roots. SCHLOTER and HARTMANN (1998) applied strain-specific fluorescently labelled monoclonal antibodies and immunogold-labelling for the localisation of *A. brasilense* strains on the surface and inside wheat roots. Furthermore, reporter genes like *NifA-lacZ* (KATUPITIYA et al., 1995), *nifH-gusA* (VANDE BROEK et al., 1993) and a bi-functional *gfp*- and *gusA*-containing mini-Tn5 transposon (XI et al., 1999) were successfully used to locate *A. brasilense* on wheat roots. However,

reporter gene constructs are only useful for model inoculation trials and monoclonal antibodies are only available for certain *A. brasilense* strains. The described oligonucleotide probes targeting the 23S (KIRCHHOF and HARTMANN, 1992) or 16S rRNA (KABIR et al., 1995) are only partly suitable for whole cell fluorescence *in situ* hybridizations and some of these probes are not specific. Therefore it was necessary to develop a new set of probes with extended applicability.

This study presents a comprehensive set of new 16S rRNA targeted oligonucleotide probes in a nested design for the secure identification of bacteria belonging to the *Azospirillum-Skermanella-Rhodocista*-cluster on different phylogenetic levels. Fluorescently labelled probes were applied for a rapid identification of isolates and *in situ* localisation of *A. brasilense* Cd on maize roots using confocal laser scanning microscopy.

## Materials and Methods

### Organisms and growth conditions

The strains of the *Azospirillum-Skermanella-Rhodocista*-cluster used in this study are listed in Table 1. Additionally, the following reference bacteria were used: *Agrobacterium rubi* DSM 6772<sup>T</sup>, *Azoarcus evansii* DSM 6898<sup>T</sup>, *Caulobacter bacteroides* ATCC 15254<sup>T</sup>, *Caulobacter crescentus* DSM 4727, *Cytophaga uliginosa* DSM 2061<sup>T</sup>, *Flavobacterium mizutaii* LMG 8340<sup>T</sup>, *Ochrobactrum anthropii* LMG 3331<sup>T</sup>, *Sphingomonas yanoikuyae* LMG 11252<sup>T</sup>, *Spingomonas paucimobilis* DSM 1098<sup>T</sup>, *Thermus aquaticus* DSM 625<sup>T</sup> and *Thermus silvanus* DSM 9946<sup>T</sup>. *Azospirillum* strains from the GSF strain collection were grown at 30 °C in 1/2 DYGS medium consisting of 0.1% glucose, 0.1% malate, 0.2% yeast extract, 0.15% peptone, 0.5% MgSO<sub>4</sub>×7H<sub>2</sub>O, and 0.15% L-glutamic acid, (pH 6.0) (RODRIGUES NETO et al., 1986, modified). All other strains were cultivated as outlined in the respective catalogues of strains (DSMZ-Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; ACM-Australian Collection of Microorganisms, Department of Microbiology, The University of Queensland, St. Lucia, Australia; ATCC-American Type Culture Collection, Manassas, VA, USA; LMG-Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium).

### Media and growth conditions

Nitrogen fixing bacteria were cultivated in semisolid nitrogen-free NFB and JNFb media with the following contents (g l<sup>-1</sup>):

**Table 1.** Origin of the *Azospirillum*, *Skermanella* and *Rhodocista* strains used in this study.

Organism	Source	Reference	Origin
<i>Azospirillum-Skermanella-Rhodocista</i> -cluster			
<i>A. amazonense</i> Y1	DSM 2787 <sup>T</sup>	MAGALHÃES et al., 1983	<i>Digitaria decumbens</i> , roots
<i>A. amazonense</i> Y6	DSM 2789	MAGALHÃES et al., 1983	<i>Pennisetum purpureum</i> roots
<i>A. amazonense</i> Y2	DSM 2788	MAGALHÃES et al., 1983	<i>Hyparrhenia rufa</i> , roots
<i>A. brasilense</i> Sp7	ATCC 29145 <sup>T</sup>	TARRAND et al., 1978	<i>Digitaria decumbens</i> , roots
<i>A. brasilense</i> Cd	DSM 1843	TARRAND et al., 1978	<i>Cynodon dactylon</i> , roots
<i>A. brasilense</i> JM 6A2	DSM 1858	TARRAND et al., 1978	maize roots
<i>A. brasilense</i> Wa3	C. CHRISTIANSEN-WENIGER	CHRISTIANSEN-WENIGER and VAN VEEN, 1991	Rhizosphere of wheat

Table 1. (Continued).

<i>A. brasilense</i> Sg1	LMG 4378	VLASSAK, 1977	<i>Sorghum bicolor</i> , root
<i>A. brasilense</i> S11	LMG 4385	VLASSAK, 1977	Soil under grass field
<i>A. brasilense</i> Sp245	J. Döbereiner	BALDANI et al.,	Wheat roots, surface sterilized
<i>A. doebereineriae</i> GSF71	DSM 13131 <sup>T</sup>	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> Ma4	DSM 13400	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> GSF21	GSF/ISE	ECKERT et al., 2001	<i>M. sacchariflorus</i> , roots
<i>A. doebereineriae</i> GSF65	GSF/ISE	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> GSF66	GSF/ISE	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> GSF67	GSF/ISE	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> GSF68	GSF/ISE	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> GSF69	GSF/ISE	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> GSF70	GSF/ISE	ECKERT et al., 2001	<i>M. sinensis</i> cv. Giganteus, roots
<i>A. doebereineriae</i> GSF76	GSF/ISE	ECKERT et al., 2001	Rhizosphere soil
<i>A. doebereineriae</i> GSFfe	GSF/ISE	ECKERT et al., 2001	Rhizosphere soil
<i>A. halopraeferens</i> Au2	LMG 7107	REINHOLD et al., 1987	<i>Leptochloa fusca</i> , roots
<i>A. halopraeferens</i> Au5	LMG 7109	REINHOLD et al., 1987	<i>Leptochloa fusca</i> , roots
<i>A. halopraeferens</i> Au10	LMG 7112	REINHOLD et al., 1987	<i>Leptochloa fusca</i> , roots
<i>A. halopraeferens</i> Au7	LMG 7110	REINHOLD et al., 1987	<i>Leptochloa fusca</i> , roots
<i>A. halopraeferens</i> Au4	DSM 3675 <sup>T</sup>	REINHOLD et al., 1987	<i>Leptochloa fusca</i> , roots
<i>A. irakense</i> KA3	LMG 10654	KHAMMAS et al., 1989	<i>Oryza sativa</i> , rhizosphere
<i>A. irakense</i> KAC5	LMG 10655	KHAMMAS et al., 1989	<i>Oryza sativa</i> , rhizosphere
<i>A. irakense</i> KBC1	DSM 11586 <sup>T</sup>	KHAMMAS et al., 1989	Rice roots
<i>A. irakense</i> Asp-1	G. Winkelmann	WINKELMANN et al., 1999	Fresh water pond
<i>A. largimobile</i>	ACM 2041 <sup>T</sup>	SKERMAN et al., 1983	Fresh water
<i>A. lipoferum</i> SpA3a	DSM 1838	TARRAND et al., 1978	Grass roots
<i>A. lipoferum</i> Sp59b	DSM 1691 <sup>T</sup>	TARRAND et al., 1978	Wheat roots
<i>A. lipoferum</i> SpRG6	DSM 1841	TARRAND et al., 1978	Wheat roots
<i>A. lipoferum</i> SpRG20a	DSM 1840	TARRAND et al., 1978	Wheat roots
<i>A. lipoferum</i> GSF1	GSF/ISE	KIRCHHOFF et al., 1997	<i>Spartina pectinata</i> , roots
<i>A. lipoferum</i> GSF5	GSF/ISE	KIRCHHOFF et al., 1997	<i>Spartina pectinata</i> , roots
<i>A. lipoferum</i> GSF15	GSF/ISE	KIRCHHOFF et al., 1997	<i>Spartina pectinata</i> , roots
<i>A. lipoferum</i> GSF26	GSF/ISE	KIRCHHOFF et al., 1997	<i>M. sacchariflorus</i> , roots
<i>Azospirillum</i> sp. 4 AZ	DSM 4834	MALIK, unpublished results	Fresh water
<i>Azospirillum</i> sp. 5 AZ	DSM 4835	MALIK, unpublished results	Fresh water
<i>Azospirillum</i> sp. A1-3	DSM 1726	MALIK and SCHLEGEL, 1980	Plant litter
<i>Azospirillum</i> sp. AM-53	DSM 1727	MALIK and SCHLEGEL, 1980	Plant litter
<i>Azospirillum</i> sp. GSF9	GSF/ISE	KIRCHHOFF et al., 1997	<i>Spartina pectinata</i> , roots
<i>Azospirillum</i> sp. GSF10	GSF/ISE	KIRCHHOFF et al., 1997	<i>Spartina pectinata</i> , roots
<i>Azospirillum</i> sp. GSF19	GSF/ISE	KIRCHHOFF et al., 1997	<i>M. sacchariflorus</i> , leaves
<i>Azospirillum</i> sp. GSF31	GSF/ISE	KIRCHHOFF et al., 1997	<i>M. sinensis</i> cv. Giganteus, stems
<i>Azospirillum</i> sp. GSFB3	GSF/ISE	This study	Banana roots
<i>Azospirillum</i> sp. GSFB4	GSF/ISE	This study	Banana roots
<i>Azospirillum</i> sp. GSFB43	GSF/ISE	This study	Banana roots
<i>Azospirillum</i> sp. GSF40	GSF/ISE	This study	<i>M. sinensis</i> cv. Giganteus, roots
<i>Azospirillum</i> sp. GSF41	GSF/ISE	This study	<i>M. sinensis</i> cv. Giganteus, roots
<i>Azospirillum</i> sp. GSF42	GSF/ISE	This study	<i>M. sinensis</i> cv. Giganteus, roots
<i>Azospirillum</i> sp. GSF43	GSF/ISE	This study	<i>M. sinensis</i> cv. Giganteus, roots
<i>Skermanella parooensis</i>	ACM 2042 <sup>T</sup>	SKERMAN et al., 1983	Fresh water
<i>Rhodocista centenaria</i>	DSM 9894 <sup>T</sup>	FAVINGER et al., 1989	Hot spring

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ACM – Australian Collection of Microorganisms, Department of Microbiology, The University of Queensland, St Lucia, Australia; ATCC – American Type Culture Collection, Manassas, VA, USA; LMG – Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; GSF/ISE – GSF-National Research Center for Environment and Health, Institute of Soil Ecology, Neuherberg, Germany. Strains Sp245, Wa3 and Asp1 were kindly provided by J. Döbereiner, EMPRAPA, Rio De Janeiro, Brazil, C. Christiansen-Weniger, Wageningen, The Netherlands and G. Winkelmann, Tübingen, Germany, respectively.

5.0 D,L-malic acid, 0.50 K<sub>2</sub>HPO<sub>4</sub>, 0.20 MgSO<sub>4</sub>×7 H<sub>2</sub>O, 1.0 NaCl, 0.02 CaCl<sub>2</sub>×2H<sub>2</sub>O, 2.0 ml bromothymol blue (0.5% solution in 0.2 M KOH), 4 ml Fe EDTA solution (1.65%), 1.0 ml vitamin solution, 2.0 ml minor element solution, 1.75 g agar, adjusted to pH 6.8 with KOH. The JNFB-medium is very similar to the NFB-medium, but contains 1.50 K<sub>2</sub>HPO<sub>4</sub>, 2.0 agar and is adjusted to pH 6.0 with KOH (DÖBEREINER, 1995). The vitamin solution contains per 100ml distilled water: 10 mg biotin, 20 mg pyridoxol-HCl. The minor element solution contains (g l<sup>-1</sup>): 0.40 CuSO<sub>4</sub>×5H<sub>2</sub>O, 0.12 ZnSO<sub>4</sub>×7H<sub>2</sub>O, 1.40 H<sub>3</sub>BO<sub>4</sub>, 1.00 Na<sub>2</sub>MoO<sub>4</sub>×2H<sub>2</sub>O, 1.50 MnSO<sub>4</sub> H<sub>2</sub>O. In addition, half-strength DYGS medium was used, containing per litre: glucose (1.0 g), malate (1.0 g), yeast extract (2.0 g), peptone (1.5 g), MgSO<sub>4</sub>×7H<sub>2</sub>O (0.5 g) L-glutamic acid (1.5 g). The pH was adjusted to 6.0.

#### Isolation of the *Azospirillum* isolates from roots of banana and *Miscanthus sinensis*

Roots of banana (*Musa* sp.) were collected in Darbhanga, Bihar, India and roots of *Miscanthus sinensis* were from the LBP (Bayerische Landesanstalt für Bodenkultur und Pflanzenbau), Freising, Germany. The roots were washed with sterile water and then crushed in 4% sucrose solution using mortar and pestle. Small vessels (10 ml volume) containing 5 ml of NFB and JNFB semisolid nitrogen-free medium (DÖBEREINER, 1995) were inoculated with serial dilutions of crushed roots. After 5 days of incubation at 30 °C one loop of pellicle forming cultures was transferred into fresh semisolid medium. Further purification was done on NFB and JNFB agar plates, supplemented with 50 mg yeast extract l<sup>-1</sup> as well as on 1/2 DYGS medium agar plates.

#### Cell fixation

Cells from exponentially growing cultures were fixed with 4% (w/v) paraformaldehyde as described by AMANN et al. (1995).

#### Plant growth and inoculation

*Zea mays* H431 cultivar seeds (provided by CIMMYT, Centro Internacional de Mejoramiento de Maiz y Trigo, Texcoco, Mexico) were surface sterilized with sixfold diluted household bleach (Chloramine T, 30%) for 15 min at 20 °C and 3 min in vacuum. The seeds were washed extensively with sterile distilled water and allowed to germinate for 3 days in sterile dish plates with wet paper in the dark at 25 °C. Axenic seedlings with approximately 1 cm length were individually and aseptically transferred to cotton plugged glass tubes (200 × 22 mm) containing sterilized sand (20 g). Tubes were put in a growth chamber with 16h/8 h day/night cycle, at constant temperature of 25 °C for 2 weeks. After 2 weeks the plants reached a height of approx. 10 cm.

For inoculation, a culture of *Azospirillum brasilense* Cd was grown in 1/2 DYGS liquid medium at 30 °C in a rotary shaker to an optical density (OD<sub>590</sub>) of 1.0. The cells were washed with a sterile NaCl solution (0.85%) and diluted to about 10<sup>7</sup> cfu/ml in sterile NaCl solution. The seedlings were carefully removed from the tubes with sand and the roots were placed into the bacterial cell suspension. After incubation for 2, 4, 6, or 8 hours at room temperature, the seedlings were rinsed by dipping them twice into sterile distilled water. The remaining water was removed from the roots by putting the roots on a sterile adsorbent towel. Afterwards, the roots were immediately cut into pieces and fixed for FISH.

#### Preparation of the root samples for FISH

14 days after inoculation, seedlings were carefully removed from the tubes and the roots were washed in sterile PBS. Root pieces (10 to 30 mm in length) were transferred to fixation buffer (4% paraformaldehyde in PBS) and fixed for at least

1 hour at 4 °C. Samples were washed and dehydrated in 50, 80 and 100% ethanol (3 min each). After being dried, the root pieces were stored at room temperature.

#### Probe design, synthesis and labelling

Probe design was performed with the PROBE DESIGN tool of the software package ARB (STRUNK and LUDWIG, 1997). Oligonucleotide probes labeled at the 5'-end with the fluorescent dyes Cy3, Cy5 and FLUOS were purchased from INTER-ACTIVA (Ulm, Germany). Probe sequences and target sites for the probes used in this study are given in Table 2.

#### Hybridizations

Hybridizations were performed on glass slides with six wells for independent positioning of the samples. Aliquots of fixed reference cells were spotted on single wells, air dried and dehydrated by passing them through an ethanol series (50, 80 and 96%, v/v) for 3 min each. Whole cell hybridizations were performed for at least 1.5 hours at 46 °C in 10 µl of a hybridization buffer containing 0.9 M sodium chloride, 0.01% sodium dodecyl sulfate (SDS), 10 mM TRIS/HCl (pH 8.0), the specific amount of formamide (Table 1) and 5 ng of fluorescently labelled probe for each well of the slide. Washing was achieved by immediate rinsing with 1 ml pre-warmed washing buffer followed by a 20 minute immersion in washing buffer at 48 °C as previously reported by NEEF et al. (1998). The washing buffer contained 20 mM Tris/HCl (pH 8.0), 0.01% SDS and depending on the hybridization stringency, between 0.056 M and 0.9 M NaCl. Slides were shortly rinsed with deionized water to remove salts. After air drying, the slides were mounted with Citifluor anti bleaching agent AF1 (Citifluor Ltd., Canterbury, UK). For some probe combinations no simultaneous hybridization was possible due to different stringencies. In these cases, the hybridization and washing was started using the probe with the higher thermal stability followed by hybridization and washing of the probe requiring lower stringency.

For the hybridization of root pieces the protocol was slightly modified. Root samples were fixed on a microscopic slide and covered with 30 µl hybridization buffer and 15 pmol of each probe. After hybridization at 46 °C for 3 h or over night the root pieces were transferred into the washing buffer for 20 min, dipped in sterile water, air dried and mounted on a microscopic slide in Citifluor.

#### Probe evaluation

*In situ* hybridization conditions for the new oligonucleotide probes were optimised by gradually increasing the formamide concentration in the hybridization buffer as previously described (AMANN et al., 1996). The formamide concentrations recommended as optimal in Table 1 are the highest concentrations that still yield good signals with the target cells and additionally allow the discrimination of non-target cells.

#### DAPI staining

For counterstaining with the DNA-binding dye DAPI (Boehringer, Mannheim) root pieces were covered with 30 µl DAPI solution (1 µg/ml in PBS) and incubated at room temperature for 10 min in the dark. After washing the samples with distilled water and air drying, the roots were mounted in Citifluor.

#### Microscopy

Epifluorescence microscopy was performed with an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with filter sets 01, 09 and 15. For the documentation of the hybridization results the confocal laser scanning microscope Zeiss LSM 510 (Jena, Germany) was used. An argon ion and a helium neon laser supplied excitation wavelengths of 488, 543, and 633 nm suitable for FLUOS, Cy3, and Cy5, respectively. A water-immersion lens

**Table 2.** rRNA-targeted oligonucleotide probes, rRNA target positions (*E. coli* numbering, Brosius et al. 1981), specificity and optimal hybridization conditions

Probe	Sequence (5'-3')	Position	% FA <i>in situ</i> <sup>a</sup>	Specificity	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338-355	0	<i>Bacteria</i>	AMANN et al., 1990
ALF1b	CGTTCGCTCTGAGCCAG	16S, 19-35	20	Alfa subclass of <i>Proteobacteria</i> , several members of delta subclass <i>Proteobacteria</i> , most spirochetes	MANZ et al., 1992
BET42a	GCCTTCCCACTTCGTTT	23S, 1027-1043	35	Beta subclass of <i>Proteobacteria</i>	MANZ et al., 1992
GAM42a	GCCTTCCCACTTCGTTT	23S, 1027-1043		Gamma subclass of <i>Proteobacteria</i>	MANZ et al., 1992
AZO440a + AZO440b	GTCATCATCGTCGCGTGC GTCATCATCGTCGCGTGC	16S, 440-457	50	<i>Azospirillum</i> spp., <i>Skermanella</i> <i>Rhodocista</i>	This study
AZOI-655	CACCATCCTCTCCGGAAC	16S, 655-672	50	Species cluster: <i>A. lipoferum</i> , <i>A. brasiliense</i> , <i>A. halopraeferens</i> , <i>A. doebereineriae</i> , <i>A. largimobile</i>	This study
Aama1250	CACGAGGTCGCTGCCCAC	16S, 1250-1267	50	<i>A. amazonense</i>	This study
Abras1420	CCACCTTCGGGTAAAGCCA	16S, 1420-1438	40	<i>A. brasiliense</i>	This study
Adoeb587	ACTTCCGACTAAACAGGC	16S, 587-604	30	<i>A. doebereineriae</i>	ECKERT et al. 2001
Adoeb94	CGTGCGCCACTGTGCCGA	16S, 94-111	30	<i>A. doebereineriae</i>	ECKERT et al. 2001
Ahalo1115	ATGGTGGCAACTGGCAGCA	16S, 1115-1133	45	<i>A. halopraeferens</i>	This study
Ahalo1249	GCGACGTCGCTTCCCACT	16S, 1249-1266	60	<i>A. halopraeferens</i>	This study
Airak1423	CACCGGCTCAGGTAAAG	16S, 1423-1440	10	<i>A. irakense</i> -cluster	This study
Airak985	TCAAGGCATGCAAGGGTT	16S, 985-1003	35	<i>A. irakense</i> -cluster	This study
Alila1113	ATGGCAACTGACGGTAGG	16S, 1113-1130	35	<i>A. lipoferum</i> , <i>A. largimobile</i>	This study
Rhodo654	ACCCACCTCTCCGGACCT	16S, 654-671	65	<i>Rhodocista centenaria</i>	This study
Sparo1402	ACTCCATGGTGTGTACGG	16S, 1402-1419	–	<i>Skermanella parooensis</i>	This study
Sparo127	GTACCAACAGGGAGGTTT	16S, 127-144	–	<i>Skermanella parooensis</i>	This study
Sparo66	CTAGGGGCGAAGGCCTCG	16S, 66-83	–	<i>Skermanella parooensis</i>	This study
Sparo84	CGTGCGCCACTAGGGGCG	16S, 84-101	20	<i>Skermanella parooensis</i>	This study
Ahalo1115C	ATGATGGCAACTGGCAGTA	16S, 1115-1133	45	Competitor	This study
Ahalo1249C	GCGACTTCGCTTCCCACT	16S, 1249-1266	60	Competitor	This study
Abras1420C	CACCTTCGGGTAAAACCA	16S, 1420-1437	40	Competitor	This study
Alila1113C	ATGGCAACTGGCGGTAGG	16S, 1113-1130	20	Competitor	This study

<sup>a</sup>Amount of formamide (% v/v) in hybridization buffer

with a magnification of  $\times 63$  was used. Image processing and analysis were performed with the standard software package provided by Zeiss. The data were converted to a postscript file with the software package Corel Draw (Version 5.0, Corel Corp., USA).

#### Tree construction

Phylogenetic analyses were performed by applying distance matrix (ARB, Felsenstein, Jukes Cantor), maximum parsimony (ARB, PHYLIP) and maximum likelihood methods (fast DNAML, MAIDAK et al. 1994). The data sets used for the calculations varied with respect to the reference sequences as well as the alignment positions included.

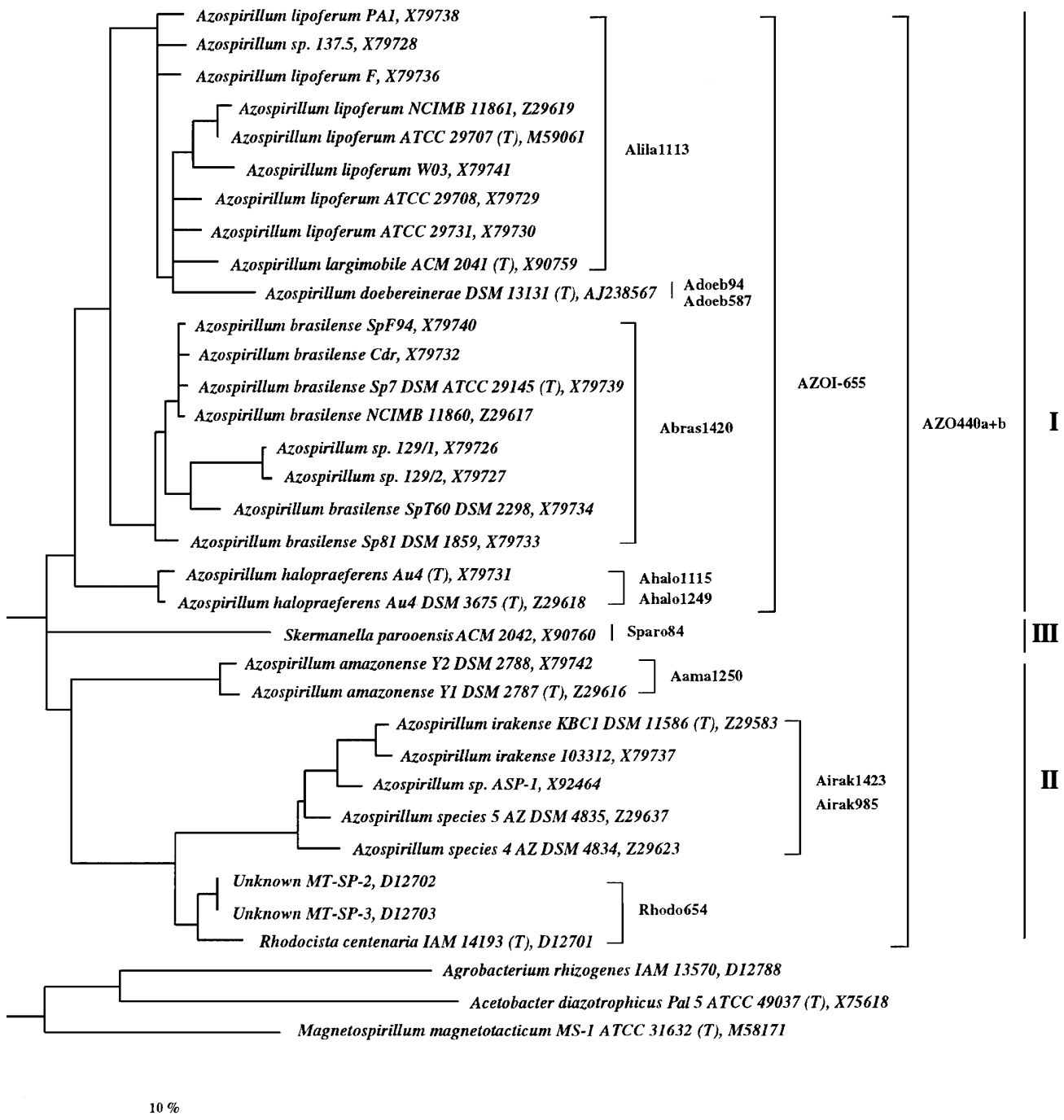
## Results and Discussion

### Phylogeny of the *Azospirilli*-cluster

The specificity of the new probes for the *Azospirillum*-*Rhodocista*-*Skermanella* cluster should reflect the phylogeny within this group. To define phylogenetically mean-

ingful groups, it was necessary to calculate a tree including all 16S rRNA sequences available for the *Azospirilli* as basis for the probe design. The tree shown in Fig. 1 is based on the results of a maximum likelihood analysis of 31 *Azospirilli* sequences (>90% complete) and 60 sequences of other  $\alpha$ -subclass proteobacteria (>90% complete). Only alignment positions which share common residues in at least 50% of all available sequences of  $\alpha$ -subclass proteobacteria were included for calculations. The tree topology was evaluated and corrected according to the results of distance and maximum parsimony analyses (LUDWIG et al., 1998).

Comparative sequence analysis revealed that the species of the genus *Azospirillum* are members of a phylogenetic subgroup of the  $\alpha$ -subclass of *Proteobacteria*. This *azospirilli*-cluster also comprises the nitrogen-fixing, non-sulfur purple phototrophic bacterium *Rhodocista centenaria* (FAVINGER et al., 1989) and *Skermanella parooensis* (formerly *Conglomeromonas parooensis* subsp. *parooensis*; LINDSAY



**Fig. 1.** Phylogenetic relationship of the species and genera belonging to the Azospirilli-cluster. The tree is based on the results of a maximum likelihood analysis of 31 *Azospirillum* sequences (>90% complete) and 60 sequences of other  $\alpha$ -subclass proteobacteria (>90% complete). Only sequence positions which share common residues in at least 50% of all available sequences from  $\alpha$ -subclass proteobacteria were included for the calculation of this tree. The tree topology was evaluated and corrected according to the results of distance and maximum parsimony analysis. Multifurcations indicate branches for which a relative order of the branching cannot unambiguously be determined or a common order is not supported applying different treeing methods. For the sake of clarity, only a selection of the reference sequences is shown. The bar indicates 10% estimated base changes.

The tree shows a summary of the newly developed 16S rRNA targeted oligonucleotide probes for the *Azospirillum*-*Skermanella*-*Rhodocista*-cluster and the two already described probes Adoeb94 and Adoeb587 (ECKERT et al. 2001). The specificity of the probes is displayed with brackets.

and STACKEBRANDT, 1999) which is unable to fix nitrogen under anaerob conditions (DEKHIL et al., 1997).

Three subclusters could be distinguished within the Azospirilli-cluster (Fig. 1): The first group comprises *A. lipoferum*, *A. largimobile*, *A. doebereinae*, *A. brasiliense* and *A. halopraeferens* (subcluster I). *A. halopraeferens* branches on a separate line within this group. The second cluster (subcluster II) is more heterogeneous as it contains *A. irakense*, *A. amazonense* and the phototroph *Rhodocista centenaria*. The third lineage is represented by *Skermanella parooensis* (subcluster III). It has to be noted that the position of this species within the azospirilli-cluster could not be determined unambiguously applying alternative treeing methods. Performing a neighbour joining analysis *Skermanella parooensis* clusters with the first Azospirilli-subgroup as described by DEKHIL et al. (1997). However, applying a maximum likelihood analysis, *Skermanella parooensis* clusters together with *A. amazonense*, *A. irakense* and *R. centenaria*. With a maximum parsimony analysis it clusters on a very short branch outside the Azospirilli lineage. Consequently, as a compromise a consensus tree was used and the inconsistency or uncertainty was visualized by multifurcation (LUDWIG et al. 1998). In the consensus tree *S. parooensis* represents a separate branch within the Azospirilli-cluster. This was supported by the fact, that *S. parooensis* – as the only member of this cluster – is unable to fix nitrogen and to form scarlet coloration of colonies on Congo red containing medium (DEKHIL et al., 1997).

#### Re-evaluation of previously published oligonucleotide probes

The 16S and 23S rRNA databases as well as the number of species known were considerably enlarged during the last years. As a consequence, probes described on a more limited dataset, no longer show the desired specificity (AMANN et al., 1996). Therefore, it is necessary to regularly check the specificity of oligonucleotide probes.

KABIR et al. (1995) described three probes for the identification of *Azospirillum* strains by radioactive colony hybridization. A recent database check using the Probe Match tool of the software package ARB (STRUNK and LUDWIG, 1997) revealed that these probes are no longer species specific. Probe Aba, for example, once designed to detect *A. brasiliense* and *A. amazonense* is also 100% complementary to *Magnetospirillum gryphiswalde*, *Leptonema illini*, *Clostridium cellulosi*, *Methanococcus aeolicus* and the recently described *A. doebereinae*. In addition, probe Ai originally targeted to the 16S rRNA of *A. lipoferum*, is not species-specific, because – according to the current sequence data – this probe is also complementary to the respective target sites of *Rickettsia akari* and *Sphingomonas adhaesiva*.

The probe Ai, originally designed for the specific detection of *A. irakense* (KABIR et al., 1995), shows identical target regions within the 16S rRNA of more than 20 other species from different phyla. According to current sequence data all three probes are complementary to members of phylogenetically not coherent groups and therefore they are not phylogenetically meaningful. Therefore, it cannot be recommended to use these probes for identification purposes.

Besides the 16S rRNA directed probes, several probes targeting the 23S rRNA of Azospirilli were described (KIRCHHOF and HARTMANN, 1992), such as the genus-specific probe AZO23S, the species-specific probes AA for *A. amazonense*, AB for *A. brasiliense*, AH for *A. halopraeferens*, AL for *A. lipoferum*, AI for *A. irakense*. A database check with about 550 published and unpublished complete and partial 23S rRNA sequences confirmed for the species-specific probes, that all non target organisms have at least two mismatches to the target organisms and therefore can be discriminated during hybridizations. Since the number of 23S rRNA sequences available from databases is still very limited the PROBE MATCH can only be a hint for the specificity and the real specificity of these probes has to be checked performing extensively hybridization experiments with both phylogenetically closely related and also distinct reference bacteria (STOFFELS et al., 1998). The specificity of the probes described by KIRCHHOF and HARTMANN (1992) were only tested with a very limited set of reference organisms and therefore further experiments would be necessary. The genus specific probe AZO23S is a mixture of four sequence variations and has only one weak mismatch with some non target organisms e. g. *Rhodobacter capsulatus* and *Bradyrhizobium japonicum*. Therefore, this probe cannot be recommended for the identification of the genus *Azospirillum* anymore. Furthermore, up to now the *in situ* accessibility of the 23S rRNA targeted probes was only shown for probe AB by ASSMUS et al. (1995). However, he also mentioned that the signal intensity of this probe is low and therefore only a limited application of this probe in environmental samples is possible.

Recently ECKERT et al. (2001) published two probes for the specific detection of a new *Azospirillum* species, *A. doebereinae*, by FISH. Both probes are targeted to the 16S rRNA and a database check revealed that they show the desired specificity.

#### Design of new oligonucleotide probes and evaluation of specific hybridization conditions

Probe design and specificity analysis were performed by using an ARB sequence database containing a total of about 10.000 complete and partial 16S rRNA sequences and the ARB-tools PROBE DESIGN and PROBE MATCH. All probe sequences, target positions, hybridization conditions and specificities are given in Table 2. An overview of the probes and their specificities is presented in Figure 1. The specificity of the probes was tested by whole cell hybridizations with fluorochrome labelled-probes and representative reference organisms (Table 3). Stringency of hybridizations were gradually increased by the addition of deionized formamide in 5% increments. The formamide concentrations recommended as optimal in Table 2 are the highest that still yield good signals with the target cells and additionally allow a discrimination of non target cells. It has to be noted, that some of the probes also showed specific hybridization signals at lower stringency. Since the database contains only a selection of bacterial 16S rRNA sequences (isolates and clones) and our current knowledge of microbial diversity is still very limited, it is advisable for most of the applications to apply the hybridization conditions as stringent as possible.





Table 3. (Continued).

Strain	ALF1b	Azo-440a+b	AZO1-655	Aama-1250	Abras-1420 + C	Adoeb-94	Adoeb-587	Ahalo-1115 + C	Ahalo-1249 + C	Airak-985	Airak-1423	Alila-1113 + C	Rhodo-654	Sparo-1402
<i>Azospirillum</i> sp. GSFB43	+	+	+	-	+	-	-	-	-	-	-	-	-	-
<i>Azospirillum</i> sp. GSF40	+	+	+	-	-	-	-	-	-	-	-	+	-	-
<i>Azospirillum</i> sp. GSF41	+	+	+	-	-	-	-	-	-	-	-	+	-	-
<i>Azospirillum</i> sp. GSF42	+	+	+	-	-	-	-	-	-	-	-	+	-	-
<i>Azospirillum</i> sp. GSF43	+	+	+	-	-	-	-	-	-	-	-	+	-	-
<i>Skermanella parooensis</i> ACM 2042 <sup>T</sup>	+	+	-	-	-	-	-	-	-	-	-	-	-	+
<i>Rhodocista centenaria</i> DSM 9894 <sup>T</sup>	+	+	-	-	-	-	-	-	-	-	-	-	+	-
Selected reference organisms														
<i>Agrobacterium rubi</i>	+	-	nd	-	-	nd	nd	-	nd	-	-	nd	nd	
<i>Agrobacterium rhizogenes</i>	+	nd	nd	nd	-	nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>Azoarcus evansii</i>	-	nd	nd	nd	nd	nd	nd	nd	nd	-	nd	nd	nd	nd
<i>Caulobacter crescentus</i>	nd	-	nd	-	-	nd	nd	-	nd	nd	-	nd	nd	-
<i>Cytophaga uliginosa</i>	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	nd	-	nd	nd
<i>Flavobacterium mizutaii</i>	nd	-	nd	nd	nd	nd	nd	nd	nd	nd	-	nd	nd	nd
<i>Ochrobactrum anthropi</i>	+	nd	nd	nd	-	nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>Sphingomonas yanoikuyae</i>	+	-	-	-	nd	nd	nd	nd	nd	-	nd	-	nd	-
<i>Spingomonas paucimobilis</i>	+	nd	-	-	-	nd	nd	-	nd	nd	nd	-	nd	Nd
<i>Thermus aquaticus</i>	nd	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>Thermus silvanus</i>	nd	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd – not determined

### Probes for the genera *Azospirillum*, *Skermanella* and *Rhodocista* (Azospirilli-cluster)

Using the PROBE DESIGN tool of the software package ARB no diagnostic region which was 100% complementary to all 16S rRNA sequences of the Azospirilli-cluster and different for all other species could be found. The 16S rRNA position 440 to 457 (*E. coli* numbering, BROSIUS et al., 1981) was most promising, which was also described by FUCHS et al. (1998) for its very good accessibility for fluorescently labelled oligonucleotide probes. The suggested probe AZO440a matches perfectly with all *A. brasilense*, *A. lipoferum*, *A. halopraeferens*, *S. parooensis* and *Rhodocista centenaria* sequences. The sequences of *A. amazonense* and some *A. irakense* strains have one weakly destabilizing G-U mispairing, whereas the *Azospirillum* strains Asp1, DSM 4835 and DSM 4834, which belong to the *A. irakense* cluster (Fig. 1), have one strongly destabilising C-A mismatch. All non target organisms have at least three mismatches. Consequently, two probes for the Azospirilli-cluster were constructed. Probe AZO440a for the detection of most of the Azospirilli strains and probe AZO440b that differ only in target position 444 for the detection of the strains Asp1, DSM 4835 and DSM 4834. The hybridization conditions for the probe AZO440a were adjusted in such a way, that *Azospirillum* strains with weakly destabilising mismatches should be detected by this

probe, whereas non-target organisms with at least three mismatches should be discriminated. The specificities of the probes were tested by whole cell hybridization with fluoro-chrome-labelled probes and representative reference organisms. Both probes AZO440a and AZO440b yielded strong hybridization signals with the target cells in the presence of up to 50% formamide in the hybridization buffer followed by a significant decline of the signal intensity at 55% formamide. Obviously, stable hybrids between the probes and the target sequences were no longer formed at higher formamide concentrations. Under stringent hybridization conditions (50% formamide) the probe AZO440a yielded good signals with all tested target organisms and, as expected, also with the *Azospirillum irakense* and *A. amazonense* strains which have one weakly destabilising G-U mispairing in the target region. *Azospirilli* strains with one strongly destabilising mismatch showed detectable but weaker hybridization signals with probe AZO440a, but strong signals with probe AZO440b. Therefore, the probes AZO440a and AZO440b should be used together at a formamide concentration of 50% for a reliable *in situ* identification of all bacteria belonging to the genera *Azospirillum*, *Skermanella* and *Rhodocista* (Azospirilli-cluster). Selected non-target bacteria, such as *Thermus silvanus*, *Magnetospirillum magnetotacti* and *Flavobacterium mizutaii* with representative mismatch constellations showed no hybridization under stringent hybridization conditions.

### Probes for the Azospirilli-subclusters

Probe AZOI-655 was designed for the detection of *A. lipoferum*, *A. largimobile*, *A. doebereinae*, *A. brasilense* and *A. halopraeferens* that form a separate subcluster within the Azospirilli-cluster (Azospirilli-subcluster I). The target site for the probe is the 16S rRNA position 655 to 672 (*E. coli* numbering, BROSIUS et al., 1981) that is identical and specific for all species of this subcluster. The discrimination of *Skermanella parooensis* relies on a G-G mismatch at position 659 (*E. coli* numbering, BROSIUS et al., 1981), whereas all other non target organisms have at least two mismatches. Binding of probe AZOI to these bacteria could be prevented by addition of at least 50% formamide to the hybridization buffer. The situation was different for the Azospirilli-subcluster II. The probe design tool was not able to identify a common 16S rRNA sequence specific for *A. irakense*, *A. amazonense* and *R. cetenaria*. This reflects the phylogenetic heterogeneity of the subcluster II. The Azospirilli-subcluster III comprises only the species *Skermanella parooensis*.

### Probes at species levels

- ***A. lipoferum*, *A. largimobile* and *A. doebereinae*:** These species are very closely related within the Azospirilli-subcluster I. Based on 16S rRNA sequence analysis no relative branching order for these three species could unambiguously be determined as reflected by a multifurcation in the tree (Figure 1). For *A. doebereinae* two probes (Adoeb94 and Adoeb587) that are accessible for *in situ* hybridizations are available (ECKERT et al., 2001). We developed the probe Alila1113 for the species cluster *A. lipoferum*-*A. largimobile*. The probe has only one central C-A mismatch with the non target sequences of *A. brasilense* strains. As described by NEEF et al. (1998) for a probe for the phylum *Planctomyces*, this mismatch constellation is insufficient for full discrimination without a competitor able to capture the 16S rRNA of these organisms. Therefore, we constructed the competitor Alila1113C to block the probe binding sites of non-target *A. brasilense* strains. At a formamide concentration of 35% and an equimolar amount of Alila1113+C, hybridization signals of *A. brasilense* cells were decreased to the low level of autofluorescence, while the *A. lipoferum* and *A. largimobile* target cells still yielded strong signals. *Bacteroides putredinis* contains only one weakly discriminating base change (U-G) at position 9 of the target region (position 121 in *E. coli* numbering). Probably, this mismatch cannot be discriminated at the applied stringency of 35% formamide and *Bacteroides putredinis* might also be detected by the probe Alila1113. However, when this probe is used in a top to bottom approach together with the here described probes AZO440a+b and/or AZOI, a misidentification is excluded. *Cytophaga uliginosa* which has two weakly destabilising mismatches (G-U and T-G) with the probe target region displayed no fluorescence signals.

- ***A. brasilense*:** The PROBE DESIGN tool suggested the 16S rRNA position 1420 to 1438 as a diagnostic region for a species-specific probe for *A. brasilense*. This region is known to be accessible for *in situ* hybridization. The *A. lipoferum*

strains ATCC 29707T, NCIMP 11861 and EBI F have only one weakly destabilising G-U mismatch at position 5 of the target region. All other non target organisms have at least 2 mismatches. Therefore, to increase the specificity of the probe, it should be used together with the competitor Abras1420C. The competitor is one nucleotide shorter than the probe Abras1420 and is 100% complementary to the *A. lipoferum* strains ATCC 29707T, NCIMP 11861 and EBI F and to *Bartonella* spp., *Phyllobacterium* spp., *Nanobacterium* sp. and *Wolbachia melophagi*. The combination of Abras1420 and Abras1420C enables reliable whole cell identification of *A. brasilense* with an addition of 45% formamide to the hybridization buffer.

- ***A. halopraeferens*:** For the design of specific probes for the species *A. halopraeferens* only the sequence of the type strain was available. After comparative sequence analysis position 1115-1133 and 1249-1266 of the 16S rRNA of *A. halopraeferens* were chosen as target sites for the probes Ahalo1115 and Ahalo1249. Both regions were suitable for whole cell hybridization. To discriminate all non-target organisms competitor oligonucleotides were designed. The competitor Ahalo1249C is complementary to all *A. lipoferum* strains and to *A. largimobile*, whereas Ahalo1115C blocks *A. doebereinae*. For good signal strength and specific hybridization conditions, probe Ahalo1249 should be used together with the competitor at 60% formamide. Probe Ahalo1115 and the competitor Ahalo1115C show the desired specificity at a formamide concentration of 45%, but the sensitivity for this probe was significantly lower than for the probe Ahalo1249. This is certainly a disadvantage for the application in environmental samples. *In situ* hybridizations with five different *A. halopraeferens* strains revealed that both probes are species-specific (Table 3). Obviously, the tested *A. halopraeferens* strains comprise a homogenous set of strains.

- ***A. amazonense*:** The probe Aama1250 was developed for the specific detection of *A. amazonense*. It is targeted to the same 16S rRNA as the probe Ahalo1249 and it also yielded bright and strong hybridization signals. All non-target bacteria have at least two mismatches to the probe sequence and can be discriminated at the recommended stringency (50% formamide).

- ***A. irakense*-cluster:** Probes Airak985 and Airak1423 are complementary to target sites identified to be specific for the *A. irakense*-cluster (16S rRNA *E. coli* positions 985-1003 and 1423-1440) by the PROBE DESIGN tool. This cluster comprises the *A. irakense* strains KBC1, 103312 and ASP-1 and the *Azospirillum* sp. strains DSM 4834 and DSM 4835. Strains DSM 4834 and DSM 4835 were deposited on the basis of their nitrogen fixing ability, morphology and physiological characteristics at the DSMZ as *Azospirillum* sp. (XIA et al., 1994). They have an overall 16S sequence similarity of 97.6 and 98.0 with the *A. irakense* type strain. Up to now it is still questionable, if they comprise an own species or not. For specific and bright hybridization probe Airak985 and Airak1423 should be used with 35% and 10% formamide in the hybridization buffer, respectively.

- ***Rhodocista centenaria*:** The genus *Rhodocista* comprises the species *Rhodocista centenaria* IAM 14193T and the

two isolates MT-Sp-2 and MT-Sp-3 (KAWASAKI et al., 1992). It represents nitrogen-fixing, phototrophic bacteria of the Azospirilli-cluster. For the specific detection of this genus the probe Rhodo654 was designed. Specific hybridization is readily achieved at the recommended formamide concentration of 65% (Table 3).

- ***Skermanella parooensis*:** To complete the probe set for the Azospirilli-cluster we also tested several diagnostic 16S rRNA regions for *S. parooensis*: Sparo1402, Sparo127, Sparo66 and Sparo84. Surprisingly, the Cy3-labelled probes Sparo1402, Sparo127 and Sparo66 yielded no detectable binding to whole fixed cells of *S. parooensis* even though the target sites for these probes were shown to be accessible at the 16S-rRNA of *E. coli* for FISH (FUCHS et al., 1998). Only with probe Sparo84, targeted to a region with a very poor *in situ* accessibility in *E. coli*, bright *in situ* hybridization signals could be achieved. These results support the conclusions by FUCHS and coworkers (1998) that the accessibility of probe target sites in other bacteria could be different to the situation in *E. coli*. The probe Sparo84 should be used at 20% formamide. At this stringency the sensitivity in whole cell hybridizations is very good and the specificity was as expected. Since *S. parooensis* AUS 2042 is the only *Skermanella parooensis* isolate described until now, more isolates will be necessary to proof, whether the probe Sparo84 is species or genus specific.

An interesting observation could be made during the hybridization experiments with *Skermanella* reference cells. *Skermanella parooensis* is described as a bacterium which exhibits unicellular and multicellular phases of growth. Unicellular phase cells are rod-shaped, with rounded or tapered ends. Multicellular conglomerates arise from single cells which lose motility, become optically refractile and reproduce by multiplanar centripetal septation. Under suitable conditions, conglomerates dissociate into single cells (DEKHIL et al., 1997; SKERMAN et al., 1983). In a para-formaldehyd fixed *Skermanella parooense* overnight culture both morphotypes could be observed. Interestingly, the multicellular aggregates yielded strong hybridization signals with the rRNA targeted, fluorescently labelled oligonucleotide probes Sparo84 and AZO440a+b whereas the single cells showed only weak or no fluorescence signals (Fig. 2 C). Obviously, cells from the multicellular growth phase are physiologically more active, because the signal intensity of *in situ* hybridizations correlate with the cellular rRNA content and growth rates of the target cells (AMANN et al., 1995). Therefore, conglomerates cannot be considered as resting stages. An alternative explanation is that the cell peripheries of the unicellular phase cells are modified and may hinder the access of oligonucleotides to their target sites. The conglomerates displayed remarkable autofluorescence, but its intensity was significantly below the probe conferred fluorescence.

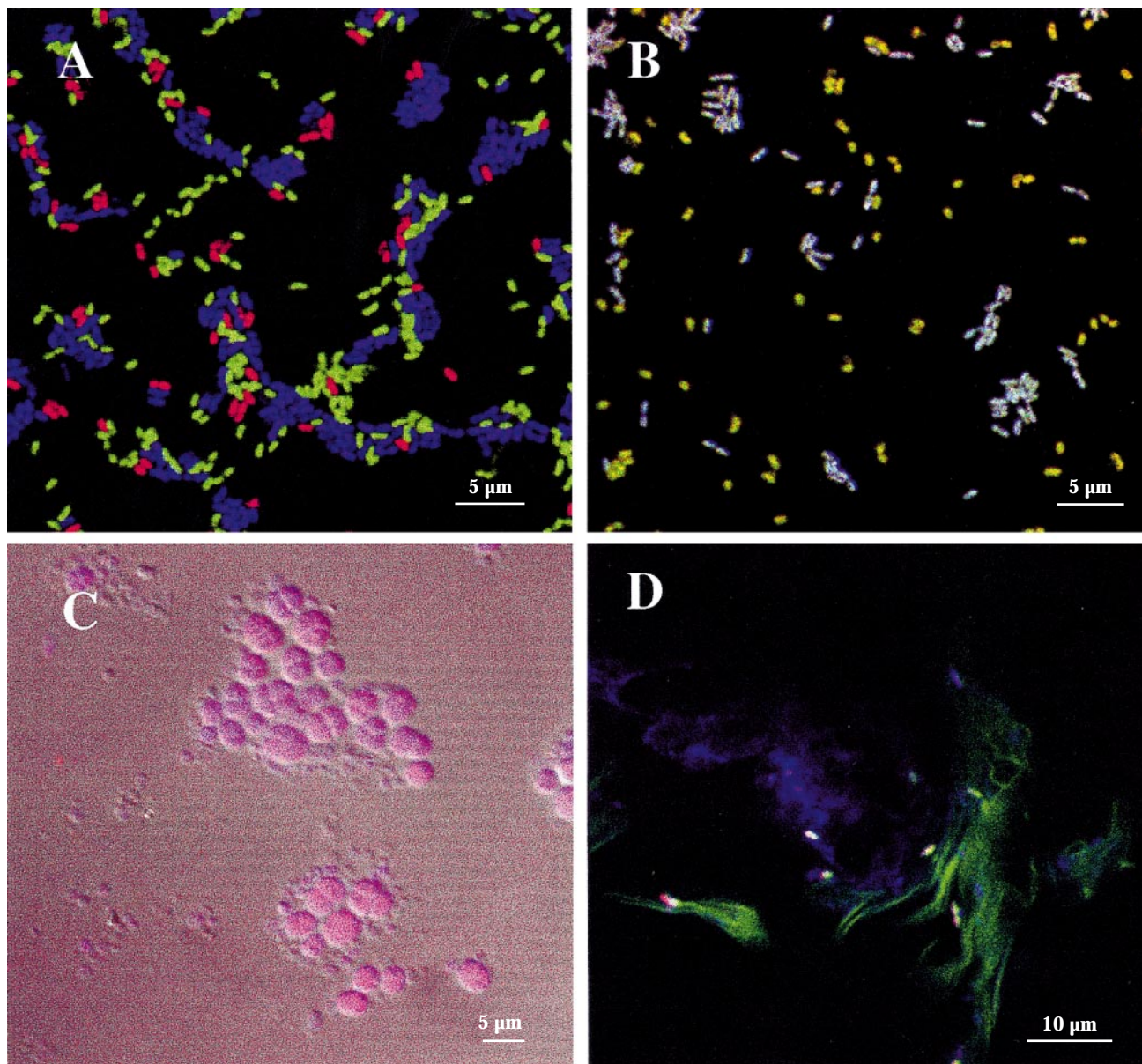
### Specificity and application of the new probes

To test the specificity of the new probes 57 strains of the *Azospirillum-Rhodocista-Skermanella*-cluster were hybridized with the complete probe set. The strain collec-

tion (Table 1) comprises a large part of Azospirilli strains available in different strain collections as well as new isolates. All strains hybridized with the genus-cluster probe AZO440a+b and, except one (GSF 31), all strains could be identified with the species or species-cluster probes (Table 3). All probes showed the desired specificity and no cross reactions of the species and species-cluster probes could be observed. The hybridization results fit very well with the description of the strains based on physiological and molecular approaches. Only for the strains GSF21, *Azospirillum* sp. DSM 1726 and *Azospirillum* sp. DSM 1727 the results differ. KIRCHHOF et al. (1997) had identified the strain GSF21 by radioactive dot blot hybridization with the 23S-rRNA targeted probe AL as *A. lipoferum*. According to our hybridization results and the previously published results from ECKERT et al. (2001) this strain should be grouped to the newly described species *A. doebereineriae*. The two strains DSM 1727 and DSM 1726 were received from the DSMZ (Braunschweig, Germany) as *Azospirillum* sp. Comparative 16S rRNA sequence analysis performed by XIA et al. (1994) revealed that the two strains were missclassified and did not cluster with the other Azospirilli. FISH with oligonucleotide probes for the alfa-, beta- and gamma-subclasses of *Proteobacteria* (MANZ et al., 1992) and the new probe set for the Azospirilli-cluster indicates that there is probably a confusion with the two strains. In our hands, DSM 1727 did not hybridize with the probe GAM42a which is specific for the gamma-subclass of *Proteobacteria*. However, it hybridized with probe ALF1b, which is specific for the alfa-subclass of *Proteobacteria*, some members of the delta subclass of *Proteobacteria* and most Spirochaetes. A further analysis revealed that the strain belongs to the alfa subclass of *Proteobacteria* but not to the Azospirilli-cluster. For the strain DSM 1726 the situation was more difficult. Obviously, it is a mixed culture of alfa- and gamma-subclass *Proteobacteria*. The cells hybridizing with the probe ALF1b also showed strong hybridization with the probes AZO440a+b, specific for the genera *Azospirillum*, *Skermanella* and *Rhodocista*, the probe AZOI-655, specific for the Azospirilli cluster I and the probe Alila-1113. Therefore the cells could be grouped to the *A. lipoferum*-*A. largomobile*-cluster. The cells belonging to the gamma subclass of *Proteobacteria* were not further characterised. To confirm these results we ordered the strains twice from the DSMZ and obtained the same hybridization results.

An example of single cell differentiation by FISH of different *Azospirillum* species is given in Fig. 2A. A mixture of *A. lipoferum* DSM 1691, *A. brasilense* DSM 1858 and *A. amazonense* DSM 2787 was hybridized with FLUOS-labelled probe Aama1250 specific for *A. amazonense*, Cy3-labelled probe Alila1113 specific for *A. lipoferum* and *A. doebereineriae* and Cy5-labelled probe Abras1420 for the detection of *A. brasilense*. FISH could clearly differentiate between the three species by giving red (*A. lipoferum*), green (*A. amazonense*) or blue fluorescence (*A. brasilense*) conferred by the probes.

The new probe set is a good tool for the fast and reliable screening of isolates by whole cell hybridization. We used it together with the probe ALF1b for the screening of 130 nitro-



**Fig. 2.** Multiple probe application in fluorescence *in situ* hybridization (FISH) and confocal scanning laser microscopy (CSLM).

A) *In situ* differentiation between an artificial mixture of *A. lipoferum* DSM 1691, *A. brasilense* DSM 1858 and *A. amazonense* DSM 2787. Cells of *A. brasilense* are stained blue by specific binding of the Cy5-labelled probe Abras1420, *A. lipoferum* cells are labelled red by specific binding of the Cy3-labelled probe Alila1113 and *A. amazonense* cells are stained green by specific binding of the FLUOS-labelled probe Aama1250.

B) Top to bottom approach for the whole cell identification of the nitrogen fixing banana isolate GSFB3. An artificial mixture of the isolate GSFB3 from banana roots, *A. lipoferum* DSM 1841 and *A. amazonense* DSM 2787 was simultaneously hybridized with the nested probe set AZO440a+b-Cy3, specific for the *Azospirillum-Rhodocista-Skermanella*-cluster, AZOI-655-FLUOS, specific for the species cluster *A. lipoferum*, *A. brasilense*, *A. largimobile* and *A. halopraefens* and the probe Abras1420-Cy5 which is specific for *A. brasilense*. Cells of the isolate GSFB3 are detected by all three probes resulting in a white staining of the cells.

C) Whole cell identification of *Skermanella parooensis* cells with different morphological appearance. The paraformaldehyde fixed overnight culture was simultaneously hybridized with the Cy3-labelled probe Sparo84, specific for *S. parooensis*, and the Cy5-labelled probe AZO440a+b, specific for the *Azospirillum-Skermanella-Rhodocista*-cluster. The phase contrast image was superimposed with the two epifluorescence panels by image analysis. The oligonucleotide probes allow the detection of both the unicellular and multicellular phases of growth.

D) *In situ* detection of *A. brasilense* Cd at the root surface of an inoculated wheat seedling, grown in a hydroponic system with quartz sand. A root sample was fixed and hybridized with the FLUOS-labelled probe EUB338, the Cy3-labelled probe ALF1b and with the Cy5-labelled probe AZO440a. *A. brasilense* Cd cells are binding all three probes and yield a white image.

gen fixing isolates from banana roots (results not shown). Performing a top to bottom approach we could identify three isolates (GSFB3, GSFB4 and GSFB43) as *A. mazonense*. All other isolates did not belong to the *Azospirilli*-cluster. Fig. 2B shows the whole cell hybridization of the isolate GSFB3, *A. lipoferum* DSM 1841 and *A. amazonense* DSM 2787 with the nested probes AZO440a+b, AZOI655 and Aama1250. Additionally, four isolates obtained from *Miscanthus sinensis* cv. Giganteus using nitrogen free-semisolid media could be assigned to the *A. lipoferum*-*A. largomobile* cluster by FISH.

An important application of the new probes is the *in situ* identification of inoculated or naturally occurring *Azospirilli* in the rhizosphere. Root samples of quartz sand-grown wheat seedlings, inoculated with *A. brasilense*, were hybridized with fluorescently labelled oligonucleotides. For the *in situ* detection of *A. brasilense* a top to bottom approach was performed using the nested probe set EUB338 (FLUOS-labelled), ALF1b (Cy3-labelled) and the new probe AZO440a (Cy5-labelled). The hybridization signals were monitored using confocal laser scanning microscope. Since *A. brasilense* cells hybridized with all three probes used, a white image is acquired, which is easily detectable in the plant tissue (Fig. 2D).

## Conclusion

The newly developed oligonucleotide probes greatly improve the existing probes for the *Azospirillum*-cluster. They can be used for whole cell and *in situ* hybridization of these bacteria. The nested design of the oligonucleotides enables a reliable and fast identification of bacteria belonging to the *Azospirillum*-*Skermanella*-*Rhodocista*-cluster on different phylogenetic levels.

Bacteria of the genus *Azospirillum* are important plant growth promoting rhizobacteria (PGPR) and are applied as inoculants. However, up to now little information is available about the survival, growth and the metabolic activity of the inoculated bacteria in the environment. The new probe set will allow to gain more fundamental insights into the fate and population dynamics of inoculants. On the basis of these results, beneficial and ecologically competent bacterial strains can be defined and the inoculation strategies may be improved. Application of rRNA-targeted probes in combination with probes specific for genes involved in the expression of phenotypic traits or together with antibodies against surface exposed or cytoplasmatic enzymes could combine phylogenetic identification with functional analysis (LUDWIG et al., 1998; ASSMUS et al., 1997). Recently LEE et al. (1999) reported the successful combination of *in situ* rRNA hybridization techniques, cryosectioning and laser scanning microscopy with microautoradiography. This technique will also be a promising opportunity for obtaining cultivation independent information about the role of indigenous or inoculated plant growth promoting bacteria in the rhizosphere.

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