

Wheat root colonization and nitrogenase activity by *Azospirillum* isolates from crop plants in Korea

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Abstract: Nitrogen-fixing bacteria were isolated from the rhizosphere of different crops of Korea. A total of 16 isolates were selected and characterized. Thirteen of the isolates produced characteristics similar to those of the reference strains of *Azospirillum*, and the remaining 3 isolates were found to be *Enterobacter* spp. The isolates could be categorized into 3 groups based on their ARDRA patterns, and the first 2 groups comprised *Azospirillum brasilense* and *Azospirillum lipoferum*. The acetylene reduction activity (ARA) of these isolates was determined for free cultures and in association with wheat roots. There was no correlation between pure culture and plant-associated nitrogenase activity of the different strains. The isolates that showed higher nitrogenase activities in association with wheat roots in each group were selected and sequenced. Isolates of *Azospirillum brasilense* CW301, *Azospirillum brasilense* CW903, and *Azospirillum lipoferum* CW1503 were selected to study colonization in association with wheat roots. We observed higher expression of β -galactosidase activity in *A. brasilense* strains than in *A. lipoferum* strains, which could be attributed to their higher population in association with wheat roots. All strains tested colonized and exhibited the strongest β -galactosidase activity at the sites of lateral roots emergence.

Key words: *Azospirillum*, acetylene reduction activity, 16S rDNA, ARDRA patterns, *lacZ* fusion.

Résumé : Des bactéries fixant l'azote ont été isolées de la rhizosphère de différentes cultures de céréales en Corée. Un total de 16 isolats ont été sélectionnés et caractérisés. Treize des isolats ont produit des caractéristiques semblables à celles de souches de référence de *Azospirillum*, et les 3 isolats restants se sont avérés être *Enterobacter* spp. Les isolats ont pu être caractérisés en 3 groupes selon leur profil de ARDRA et les 2 premiers groupes renfermaient *Azospirillum brasilense* et *Azospirillum lipoferum*. L'activité de réduction de l'acétylène (ARA) de ces isolats fut déterminée pour des cultures libres et en association avec des racines de blé. Il n'y eut aucune corrélation entre l'activité nitrogénase des cultures pures par rapport à celles associées aux plantes chez les différentes souches. Les isolats qui ont démontré les activités nitrogénase les plus élevées en association avec les racines de blé dans chaque groupe ont été sélectionnés et séquencés. Les isolats de *Azospirillum brasilense* CW301, *Azospirillum brasilense* CW903 et *Azospirillum lipoferum* CW1503 ont été sélectionnés pour étudier la colonisation en association avec les racines de blé. Nous avons observé une expression plus élevée de l'activité β -galactosidase chez les souches de *A. brasilense* comparativement aux souches de *A. lipoferum*; ceci pourrait être attribué à leur population plus élevée en association avec les racines. Toutes les souches analysées ont colonisé et démontré une activité β -galactosidase maximal aux sites d'émergence des racines latérales.

Mots clés : *Azospirillum*, activité des réductions de l'acétylène, ADNr 16S, profils de ARDRA, fusion *lacZ*.

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Introduction

Azospirillum spp., isolated from various geographical regions of the world, represent the best-characterized genus of plant growth-promoting rhizobacteria (PGPR) (Bashan and Holguin 1997). The genus contains 7 species viz., *Azospirillum brasilense*, *Azospirillum lipoferum*, *Azospirillum*

amazonense, *Azospirillum halopraeferens*, *Azospirillum largimobile*, *Azospirillum irakense*, and *Azospirillum doebereineriae* (Tarrand et al. 1978; Magalhães et al. 1984; Reinhold et al. 1987; Ben Dekhil et al. 1997; Khammas et al. 1989; Eckert et al. 2001). Members of the genus *Azospirillum* are Gram-negative to Gram-variable, have a curved-rod shape, and are motile by a single polar flagellum in liquid media and by polar and

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Table 1. Biochemical characteristics of the N₂-fixing strains from the rhizosphere of different crops.

Strains	Host plant	Gram reaction	Oxidase reaction	Potato infusion (BMS) agar*	Carbon source utilization			Biotin requirement	Group [†]
					Sucrose	Glucose	Malic acid		
<i>Azospirillum brasilense</i> Sp7 [‡]	—	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW5	Tobacco	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW202	Rice	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW301	Wheat	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW307	Wheat	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW406	Soybean	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW705	Rice	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW716	Onion	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW805	Onion	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW903	Taro	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW1401	Soybean	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW1402	Rice	—	+	Pink	—	—	+	—	I
<i>Azospirillum brasilense</i> CW1502	Apple	—	+	Pink	—	—	+	—	I
<i>Azospirillum lipoferum</i> 687 [‡]	—	—	+	Pink	—	+	+	+	II
<i>Azospirillum lipoferum</i> CW1503	Sudan grass	—	+	Pink	—	+	+	+	II
<i>Enterobacter</i> sp. strain CW302	Wheat	—	+	Yellow	—	+	+	+	III
<i>Enterobacter</i> sp. strain CW309	Wheat	—	+	Yellow	—	+	+	+	III
<i>Enterobacter</i> sp. strain CW902	Rice	—	+	Yellow	—	+	+	+	III

Note: BMS, bacitracin mitis salivarius.

*Typical colored colonies on potato infusion (BMS) agar were observed after incubation at 30 °C for 7 d (Krieg and Döbereiner 1984).

[†]The groupings are based on the similarities to the reference strains *A. brasilense* Sp7 and *A. lipoferum* 687. Group III differed from the reference strains and hence grouped separately.

[‡]Reference strains were obtained from the collection of Peter B. New, University of Sydney (Australia).

lateral flagella on solid media. They are oxidase positive, contain poly- β -hydroxybutyrate granules and exhibit acetylene-reduction activity (ARA) under micro-aerophilic conditions. They also produce a rising pellicle in semi-solid nitrogen-free (NFB) media. These bacteria are known to associate with the roots of wheat, tropical grasses, maize, and other cereals (Okon and Hadar 1987; Lindberg and Granhall 1984; Lalande et al. 1989; Hebbbar et al. 1991; Oh et al. 1999). *Azospirillum* spp. have been identified mainly as rhizosphere bacteria, and their mechanism of colonizing the rhizosphere has been studied by various researchers (Holguin et al. 1999; Steenhoudt and Vanderleyden 2000). The ability to form cysts and to flocculate has been linked

with the ability of *Azospirillum* to colonize the surfaces of wheat roots (Katupitiya et al. 1995; Pereg-Gerk et al. 1998). Colonization may involve a non-specific interaction between the bacteria and the plant in the form of non-specific chemotaxis that is mediated by motility (Vande Broek et al. 1993; Steenhoudt and Vanderleyden 2000). The polar flagellum is also believed to play a role in bacterial attachment as well as motility (Croes et al. 1991; Michiels et al. 1991; Steenhoudt and Vanderleyden 2000).

Arsene et al. (1994) demonstrated that bacteria carrying the reporter gene *lacZ* (β -galactosidase) could be used to estimate the extent of colonization. Recent reports address the colonization patterns of wheat by *Azospirillum brasilense*

Table 2. Bacterial strains and plasmids used.

Strain or plasmid	Reference/source
<i>Azospirillum</i> strains	
<i>A. brasilense</i> Sp7	Tarrand et al. 1978
<i>A. brasilense</i> CW301	This work
<i>A. brasilense</i> CW903	This work
<i>A. lipoferum</i> 687	Peter B. New, University of Sydney, Australia
<i>A. lipoferum</i> CW1503	This work
Plasmids	
<i>pLA-lacZ</i> *	Arsene et al. 1994

**lacZ-Kan* cartridge cloned into *pLA2917* vector^b, Tc^r, Km^r, Lac^s. *pLA2917* is a low-copy-number, broad-host-range cloning-vector derivative of RK2 that is stable in *Azospirillum* strains.

strains carrying *lacZ* fusions (Pereg-Gerk et al. 2000). The benefits of using *Azospirillum* as biofertilizers for crop plants have been well documented (Okon and Kapulnik 1986; Sarig et al. 1986; Burdman et al. 1997), and the association between *Azospirillum* and plant roots has been reported to increase the efficiency of applied fertilizers (Okon and Labandera-Gonzalez 1994; Kennedy et al. 1997; Gunarto et al. 1999). Therefore, the isolation of *Azospirillum* spp. capable of high rates of N₂ fixation and suitable for use as biofertilizers is potentially of great importance to modern agriculture.

This study aimed to isolate and identify efficient nitrogen-fixing strains of *Azospirillum* spp. from the roots of various plants from Chungbuk province, South Korea, and studied their ability to colonize wheat seedlings and fix nitrogen under rhizosphere conditions.

Materials and methods

Isolation and characterization of N-fixing isolates

Isolation of N-fixing organisms from the rhizosphere of different crops (Table 1) was carried out by the enrichment culture technique (Day and Döbereiner 1976) using semi-solid malate medium (NFB) (Baldani and Döbereiner 1980) supplemented with 50 mg·L⁻¹ yeast extract (Difco Laboratories, Detroit, Mich.). The isolates showing characteristic sub-surface pellicle formation were selected and further purified by streaking on Congo red agar medium (Rodríguez Cáceres 1982). The isolates obtained were further characterized. Cell morphology and mobility were examined under a light microscope. The biochemical tests like oxidase, Gram staining, and substrate utilization using malate medium and API 20E test strips (Bio Merieux, Marcy l'Etoile, France) were carried out for genus identification. For species identification, glucose assimilation in NFB medium and biotin requirement for growth were determined (Tarrand et al. 1978).

Acetylene-reduction assay

The acetylene-reduction assay (ARA) was performed on free-living cultures as well as on cultures in association with wheat plants. Ethylene formation was measured using a Varian model 3700 Gas chromatograph equipped with a flame ion-

ization detector and 1.8 m Porapak T column (Model HP 6890).

The ARA for free-living cultures was carried out in accordance with Han and New (1998). In brief, overnight-grown cultures were used for inoculating NFB medium and incubated for 48 h at 30 °C. The gas phase was replaced with an acetylene–air–nitrogen mixture (10:10:80 by volume) giving a reduced partial pressure of oxygen. The rate of ethylene production was measured by taking samples after 24 h. The protein concentration was determined by a modified Lowry method (Lowry et al. 1951) with bovine serum albumin (BSA) as standards.

Wheat (*Triticum aestivum*) 'Bowerbird' plants grown in sterile vermiculite were inoculated with bacteria grown overnight in NFB medium supplemented with 0.05% yeast extract. ARA was performed after 2 weeks, and the viable bacterial counts on roots were determined (Miles and Misra 1938).

PCR amplification, restriction, and sequencing of bacterial 16S rDNA

Bacterial DNA was extracted according to Sambrook et al. (1989). The amplification of 16S rDNA was performed in a 50 µL final volume containing 10 µL of total DNA, 0.2 mmol·L⁻¹ of 27F primer (5'-AGAGTTTGATCCTGG CTCAG-3', positions 8–27 in *E. coli* numbering), 0.2 mmol·L⁻¹ of 1512R primer (5'-ACGGCTACCTTGTACGACT-3', positions 1512–1493 in *E. coli* numbering) (Devereux and Willis 1995), 10 µL of 2.5 mmol·L⁻¹ of each dNTP, and 0.05 U of *Taq* DNA polymerase. A negative control (PCR mixture without DNA) was included in all PCR experiments. The reaction conditions were as follows: 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and primer extension at 72 °C for 2 min; followed by a final extension at 72 °C for 5 min. The reaction products were separated by running 5 µL of the PCR reaction mixture in 1.2% (w/v) agarose gel, and the bands were stained with ethidium bromide. The amplified ribosomal DNA restriction analysis (ARDRA) was performed to compare and detect the homology patterns of the isolates as well as known strains used as references (*A. brasilense* Sp7 and *A. lipoferum* 687). Restriction enzyme *Rsa*I was used to digest the amplified DNA to distinguish *Azospirillum* species. Amplified DNA was analyzed on a 2% agarose gel (Bandi et al. 1994; Grifoni et al. 1995), and the samples were sequenced commercially at Supamac Inc., Sydney, Australia.

Conjugation of reporter genes to *Azospirillum*

Five strains of *Azospirillum*, including reference strains, were selected for conjugation with *Escherichia coli* S17.1 carrying the plasmid *pLA-lacZ* to study colonization (Arsene et al. 1994). The plasmid and the strains used are listed in Table 2.

Conjugation was performed between *E. coli* S17.1 containing the plasmid and *Azospirillum* strains as described by Simon et al. (1983). The donor strain, *E. coli* S17.1, was grown in Luria–Bertani (LB) broth supplemented with 5 µg tetracycline·mL⁻¹. The recipients, *Azospirillum* strains, were grown in NFB liquid medium supplemented with ammo-

Fig. 1. The amplified ribosomal DNA restriction analysis (ARDRA) patterns of amplified 16S rDNA of 16 strains digested with restriction enzyme *Rsa*I. Lane M, molecular weight marker; Lane 1, *Azospirillum brasilense* Sp7; Lane 2, *Azospirillum brasilense* CW805; Lane 3, *Azospirillum brasilense* CW705; Lane 4, *Azospirillum brasilense* CW307; Lane 5, *Azospirillum brasilense* CW1402; Lane 6, *Azospirillum brasilense* CW202; Lane 7, *Azospirillum brasilense* CW1503; Lane 8, *Azospirillum brasilense* CW716; Lane 9, *Azospirillum brasilense* CW301; Lane 10, *Azospirillum brasilense* CW309; Lane 11, *Azospirillum brasilense* CW302; Lane 12, *Azospirillum brasilense* CW902; Lane 13, *Azospirillum brasilense* CW1401; Lane 14, *Azospirillum brasilense* CW1502; Lane 15, *Azospirillum brasilense* CW406; Lane 16, *Azospirillum brasilense* CW903; Lane 17, *Azospirillum brasilense* CW5; and Lane 18, *Azospirillum lipoferum* 687.

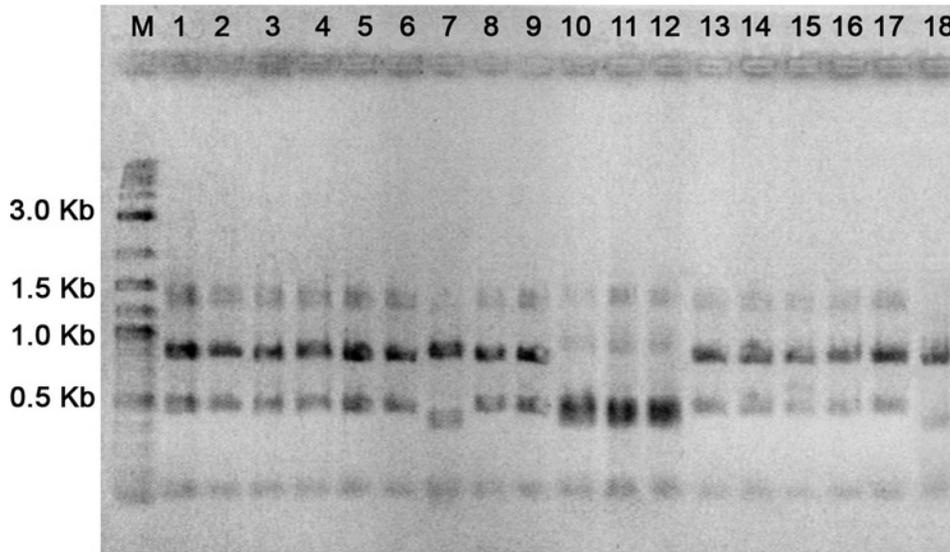


Fig. 2. Nitrogenase activity of isolates in pure culture in semi-solid nitrogen-free (NFB) medium and in association with the roots of wheat seedlings. Reference strains are *Azospirillum brasilense* Sp7 and *Azospirillum lipoferum* 687. Error bars represent SD.

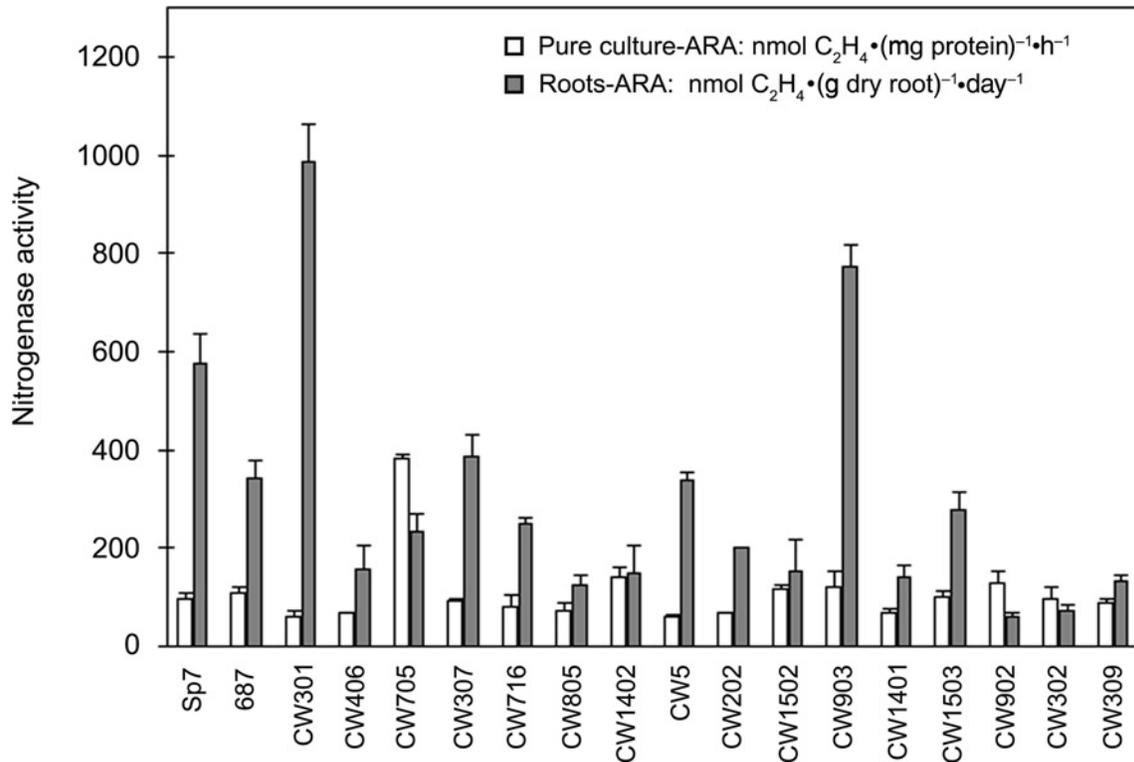


Fig. 3. β -Galactosidase activity of the *Azospirillum* transconjugants in pure cultures. Sp7, *A. brasilense* reference strain; CW301 and CW903, *A. brasilense* strains; and CW1503, *A. lipoferum* strain. Error bars represent SD.

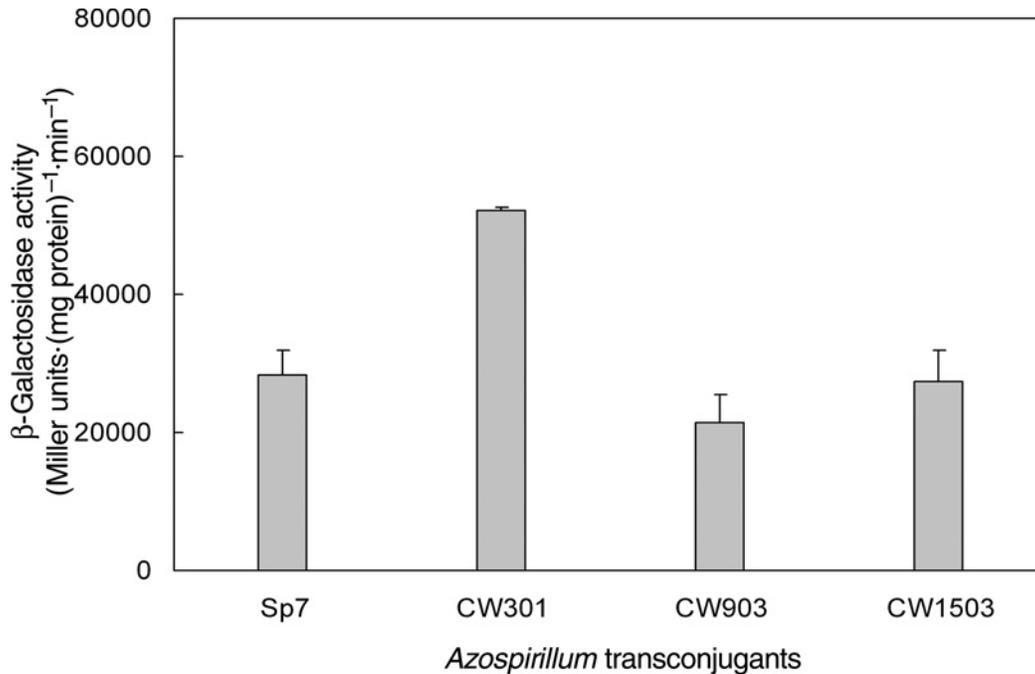


Table 3. Percent homology 16S rDNA sequences.

Strain	Organism with most similar sequence	Similarity (%)	Acc. No.*
CW301	<i>Azospirillum brasilense</i>	99	AY518780
CW903	<i>Azospirillum brasilense</i>	99	AY518777
CW1503	<i>Azospirillum lipoferum</i>	98	AY518779
CW309	<i>Enterobacter</i> sp.	99	AY518778

*Acc. Nos. obtained from the GenBank National Center for Biotechnology Information (NCBI).

nium chloride (1 g·L $^{-1}$). Conjugation took place on nutrient agar (NA; Difco) by mixing equal volumes of donor and recipient. Transconjugants were selected on minimal lactate medium (Dreyfus et al. 1983) supplemented with ammonium chloride (1 g·L $^{-1}$) and tetracycline (5 μ g·mL $^{-1}$).

Cultivation of plants and inoculation of seedlings with bacteria

Wheat seeds ('Bowerbird') that were surface sterilized and germinated on yeast extract mannitol agar (YEMA; in g·L $^{-1}$: 1.0 yeast extract, 10.0 mannitol, 0.5 K $_2$ HPO $_4$, 0.20 MgSO $_4$ ·7H $_2$ O, pH 6.8) at 30 °C were transferred aseptically to tubes containing 15 mL of N-free hydroponic solution as described by Zeeman et al. (1992). Plants were incubated in a growth chamber with a day night cycle of 14 h (27 °C) and 10 h (18 °C). After a week of growth, the hydroponic solution was inoculated with 0.2 mL of overnight grown cultures (A_{600} = 0.5). Uninoculated plant tubes and tubes inoculated with bacteria without *lacZ* fusion were routinely included as controls. In situ colonization with X-gal (5-bromo-4-

chloro-3-indolyl- β -galactoside) and β -galactosidase activity were determined 10 d after inoculation.

Assay for β -galactosidase activity and in situ detection in root segments inoculated with *Azospirillum*

Expression of the *lacZ* gene in transconjugants was assayed by measuring the ability to hydrolyze *o*-nitrophenyl- β -D-galactopyranoside (ONPG; Sigma Chemical Co. St. Louis, Mo.) (Miller 1972). Overnight-grown liquid cultures of cells containing *lacZ* fusions were used for the determination of β -galactosidase activity, and the results are expressed in Miller units·min $^{-1}$ ·(mg of protein) $^{-1}$. Root pieces from the seedlings were ground with 2 mL of Z-buffer (in g·L $^{-1}$: 0.75 KCl, 0.25 MgSO $_4$ ·7H $_2$ O, 21.49 Na $_2$ HPO $_4$, 0.24 NaH $_2$ PO $_4$ ·H $_2$ O, pH 7.4) devoid of β -mercaptoethanol. The plant-endogenous β -galactosidase activity was inactivated by heating the suspension for 15 min at 50 °C. The suspension was stabilized by the addition of 5 μ L of β -mercaptoethanol, and the bacterial cells were lysed by adding 20 μ L of 0.1% sodium dodecyl sulfate and 40 μ L of chloroform and then vortexing. The β -galactosidase activity was measured by the previously mentioned procedure (Katupitiya et al. 1995). The results are expressed as Miller units·min $^{-1}$ ·(mg of plant protein) $^{-1}$. The protein concentration of the samples was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Mississauga, Ont.) (Gogstad and Krutnes 1982).

In situ visualization of azospirilla bearing *lacZ* fusion was obtained by staining the root pieces with X-gal in accordance with Arsene et al. (1994). Root segments (2 cm long) were fixed with 1 mL of 2% glutaraldehyde solution in Z-buffer, washed, and then stained with X-gal solution. After staining, the root segments were washed 3 times in

Fig. 4. β -Galactosidase activity of wheat inoculated with *Azospirillum* transconjugants. The β -galactosidase activity was determined 10 d post inoculation. The data are averages of 3 independent experiments and error bars represent SD. Sp7, *A. brasilense* reference strain; CW301 and CW903, *A. brasilense* strains; and CW1503, *A. lipoferum* strain.

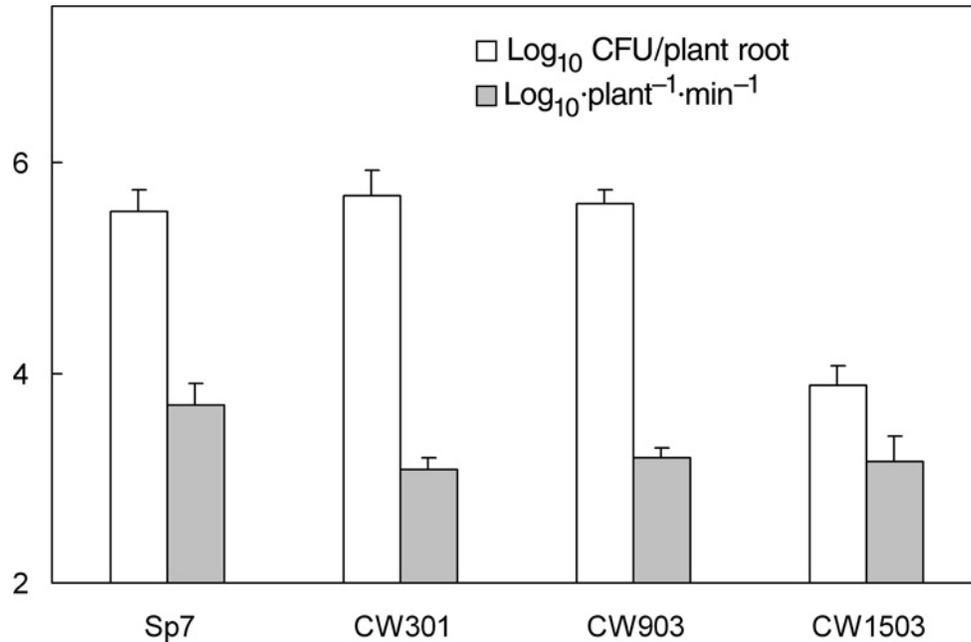
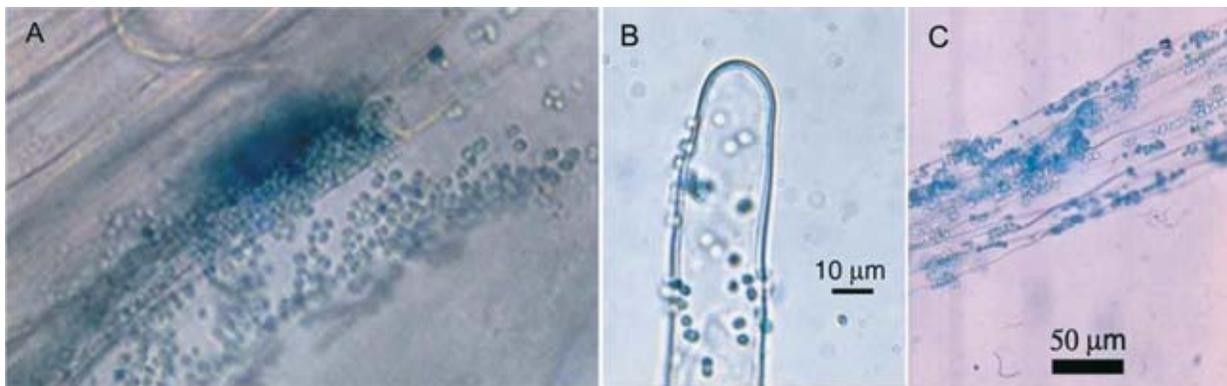


Fig. 5. In situ detection of β -galactosidase activity on root segments. All samples were stained after 10 d. X-gal staining was performed on five or six 1 cm root segments excised from the same plant starting from the tip. (A) Root inoculated with *A. brasilense* CW903 (X60); (B) Root inoculated with *A. brasilense* CW301 (X100); and (C) Root inoculated with *A. brasilense* Sp7 (X40).



Z-buffer followed by 2 washes with distilled water for 5 min and examined by light microscopy for bacterial colonization patterns.

Results and discussion

Bacteria of the genus *Azospirillum* are micro-aerophilic, N_2 -fixing Gram-negative rods, widely distributed in soil and are associated with the roots of forage crops, cereals, and non-gramenous plants (Bashan and Holguin 1997). Upon inoculation, bacteria adsorb to roots, proliferate on the surface, and may subsequently invade and colonize the internal tissues of the roots. In this study, we attempted to isolate N_2 -fixing *Azospirillum* isolates from the rhizosphere of different crops and tested their N_2 -fixation ability in association with wheat crop. We also studied the root coloniza-

tion of *Azospirillum* strains in wheat crop using the reporter genes.

A total of 16 strains isolated from the rhizosphere of different plants grown in soil from different regions of Korea that were able to fix nitrogen were selected. These strains were isolated from a variety of field-grown plants, including rice, wheat, Sudan grass, and onion, as well as several dicotyledonous plants, indicating that these nitrogen-fixing bacteria are inhabitants of the rhizosphere of many plant species. The strains were subjected to biochemical tests to confirm their identity and compared with 2 reference strains of *Azospirillum*, *A. lipoferum* 687 and *A. brasilense* Sp7. All strains were Gram-negative, oxidase positive, and formed a characteristic sub-surface pellicle in NFB medium. Of the 16 strains, 12 strains showed characteristics of *A. brasilense* Sp7 and 1 strain (CW1503) showed characteristics of

A. lipoferum 687. Three isolates viz., CW302, CW309, and CW902 showed characteristics of neither *A. lipoferum* 687 nor *A. brasilense* Sp7. Although these strains were able to use glucose and biotin for their growth, they produced typical yellow colonies on bacitracin mitis salivarius (BMS) agar (Table 1). Further analysis of these strains using the API 20E kit revealed their identity as *Enterobacter* spp. *Enterobacter* strains from potato tubers were all Gram-negative, oxidase negative, motile via flagella, and were able to fix nitrogen (Pishchik et al. 1998). *Azospirillum* had previously been isolated from the roots of various plants including cereals, legumes, vegetables, and flowering plants (Döbereiner and Day 1976; Tyler et al. 1979; Wong et al. 1980; Bally et al. 1983; Ladha et al. 1987; Cavalcante and Döbereiner 1988; De Coninck et al. 1988; Bashan 1991). In this study, *A. brasilense* was found to be the dominant species of *Azospirillum* on plant roots, and the possible variations might be due to selectivity of the host plant (Baldani and Döbereiner 1980). The findings are consistent with those of Han and New (1998), who found regional differences in the occurrence of the 2 species.

The species of *Azospirillum* can be distinguished according to their carbon source utilization (Hartmann and Zimmer 1994) and ARDRA patterns (Grifoni et al. 1995; Han and New 1998). 16S rDNA was amplified from the 16 isolates and from the 2 species of *Azospirillum* (*A. lipoferum* 687 and *A. brasilense* Sp7) that were used as the reference strains. Restriction endonuclease *Rsa*I was used to digest the amplified DNA, and the ARDRA patterns of the known members were typical. With 4 exceptions, all the other isolates produced ARDRA patterns typical of *A. brasilense* Sp7 and strain CW1503 showed patterns similar to *A. lipoferum* 687. The other 3 isolates could not be allocated to *Azospirillum* species on the basis of their ARDRA patterns (Fig. 1). The ARDRA patterns correlated well with the groupings based on carbon source utilization. ARDRA is a useful tool for the rapid screening of an *Azospirillum* population, and by using different restriction enzymes, this method could be extended to the identification of *Azospirillum* strains at the subspecies level (Grifoni et al. 1995).

There was wide variation in nitrogenase activity among the different isolates selected, ranging from 187 to 387 nmol $C_2H_4 \cdot (mg \text{ protein})^{-1} \cdot h^{-1}$, and there was no correlation between the ARA of strains in pure culture and their performance on wheat plants (Fig. 2). In association with wheat roots, 2 *A. brasilense* strains CW301 (from wheat) and CW903 (from taro) had the highest ARA measured after 48 h of incubation. This indicates that there were differences between the ability of strains to fix N_2 in pure culture and their ability to do so in association with wheat roots. *Azospirillum brasilense* CW301, originally isolated from wheat roots, had the highest ARA in association with the roots of wheat plants, but had one of the lowest values in pure culture. Little work has been done on azospirilla–host plant specificity, and the basis for any such specificity by azospirilla in associating with the host plant is unknown (Katupitiya et al. 1995).

The representative strains that showed the highest nitrogenase activity in wheat roots viz., *A. brasilense* CW301, *A. brasilense* CW903, *A. lipoferum* CW1503, and *Enterobacter* sp. strain

CW309, were selected and their identifications were confirmed by 16S rDNA sequencing. These strains were found to have 98% to 99% homology with *A. brasilense*, *A. lipoferum*, and *Enterobacter* sp., respectively, and were assigned the GenBank accession numbers AY518780, AY518777, AY518779, and AY518778, respectively, by NCBI (Table 3). Nitrogenase activities of the 3 *Enterobacter* strains in association with wheat roots were low when compared with the other strains. So for further colonization studies, *A. brasilense* CW301, *A. brasilense* CW903, and *A. lipoferum* CW1503 were used.

Conjugation of selected strains of *Azospirillum* with *E. coli* S17.1 carrying the plasmid *pLA-lacZ* was performed, and the expression of the *lacZ* gene in the conjugated strains was confirmed by the β -galactosidase assay. All the transconjugants showed considerable β -galactosidase activity under aerobic growth and the transconjugant of *A. brasilense* CW301 exhibited the highest activity followed by *A. lipoferum* CW1503 (Fig. 3). These conjugates were used for the colonization studies on wheat roots. It has been previously shown that soon after inoculation, the bacteria adhere to the roots of wheat plants and probably do not remain in the hydroponic solution (Zeman et al. 1992). Therefore, sufficient time was allowed for the bacteria to differentiate completely during the 10 d following inoculation. As a general rule, β -galactosidase activity and X-gal staining with fully or partially constitutive fusions could be correlated with the extent of colonization (Arsene et al. 1994; Vande Broek et al. 1993). β -Galactosidase activity was found to be correlated with the bacterial population associated with plant roots. Higher expression of β -galactosidase activity was observed with *A. brasilense* strains compared with the *A. lipoferum* strain, in agreement with the observed frequency of the number of cells (Fig. 4). This could reflect the adaptability of *A. brasilense* strains to the particular plant culture conditions. In a previous study, *A. lipoferum* strains were found to be more efficient than *A. brasilense* strains on the same wheat genotype (Katupitiya et al. 1995).

Bashan (1986) showed that *A. brasilense* cells migrate toward wheat roots in sand culture and in wet soil. *Azospirillum* strains have been shown to develop significant chemotactic ability towards several components. Examination of segments of wheat roots stained with X-Gal, readily enabled visualization of *Azospirillum* cells bearing *pLA-lacZ* plasmids (Fig. 5). Root colonization of hydroponically grown wheat seedlings by the *lacZ* carrying *Azospirillum* strains was detected by localized blue coloration around bacterial cells in roots stained with X-gal, undetected in control or roots inoculated with parent strains lacking a *pLA-lacZ* plasmid. The bacteria were hardly stained on the root surface. Bacteria were found mainly in groups or appeared singly on the root as a mixture of round, ovoid and comma-shaped cells. All tested strains colonized and exhibited strongest β -galactosidase activity at the sites of lateral root emergence and the area of highest root hair density near the root tip. Studies on the colonization of wheat roots using *nodG-lacZ* fusions expressing the *lacZ* gene at the highest level indicated the localization of bacteria on the young parts of roots as a thick coat (Katupitiya et al. 1995). The mechanisms responsible for the preferential colonization at

the sites of lateral root emergence and in the root hair zones may involve chemotaxis and (or) specific attachment (Vande Broek et al. 1993).

In this report, the strain with the highest nitrogenase activity in association with roots of the wheat plants was *A. brasilense* CW301, indicating that this strain may be especially suitable for inoculating wheat plants for yield improvement. However, further studies are needed to examine the efficacy of colonization of other plants with the bacterial strains.

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References

- Arsene, F., Katupitiya, S., Kennedy, I.R., and Elmerich, C. 1994. Use of *lacZ* fusions to study the expression of *nif* genes of *Azospirillum brasilense* in association with plants. *Mol. Plant-Microbe Interact.* **7**: 748–757.
- Baldani, V.L.D., and Döbereiner, J. 1980. Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* **12**: 433–439.
- Bally, R., Thomas-Bauzon, D., Heulin, T., and Ballandreau, J. 1983. Determination of the most frequent N₂-fixing bacteria in a rice rhizosphere. *Can. J. Microbiol.* **29**: 881–887.
- Bandi, C., Damiani, G., Magrassi, L., Grigolo, A., Fani, R., and Sacchi, L. 1994. Flavobacteria as intracellular symbionts in cockroaches. *Proc. Roy. Soc. Lond. Brit.* **257**: 43–48.
- Bashan, Y. 1986. Migration of the rhizosphere bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* towards wheat roots in the soil. *J. Gen. Microbiol.* **132**: 3407–3414.
- Bashan, Y. 1991. Air-borne transmission of the rhizosphere bacterium *Azospirillum*. *Microb. Ecol.* **22**: 257–269.
- Bashan, Y., and Holguin, G. 1997. *Azospirillum*-plant relationships: environmental and physiological advances. (1990–1996). *Can. J. Microbiol.* **43**: 103–121.
- Ben Dekhil, S., Cahill, M., Stackrandt, E., and Sly, L.I. 1997. Transfer of *Conglomeromonas largomobilis* subsp. *largomobilis* to the genus *Azospirillum* as *Azospirillum largomobile* comb. nov., and elevation of *Conglomeromonas largomobile* subsp. *parooensis* to the new type species of *Conglomeromonas*, *Conglomeromonas parooensis* sp. nov. *Syst. Appl. Microbiol.* **20**: 72–77.
- Burdman, S., Kigel, J., and Okon, Y. 1997. Effects of *Azospirillum brasilense* on nodulation and growth of common bean (*Phaseolus vulgaris* L.). *Soil Biol. Biochem.* **29**: 923–929.
- Cavalcante, V.A., and Dreiner, J. 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant Soil*, **108**: 23–31.
- Croes, C., Bastelaere, V., DeClercq, E., Eyers, M., Vanderleyden, J., and Michiels, K. 1991. Identification and mapping of *loci* involved in motility, adsorption to wheat roots, colony morphology, and growth in minimal medium on the *Azospirillum brasilense* Sp7 90-Mda plasmid. *Plasmid*, **26**: 83–93.
- Day, J.M., and Döbereiner, J. 1976. Physiological aspects of N₂-fixation by a *Spirillum* from *Digitaria* roots. *Soil Biol. Biochem.* **8**: 45–50.
- De Coninck, K., Horemans, S., Rombauts, S., and Vlassak, K. 1988. Occurrence and survival of *Azospirillum* spp. in temperate regions. *Plant Soil*, **110**: 213–218.
- Devereux, R., and Willis, S.G. 1995. Amplification of ribosomal RNA sequences. In *Molecular microbial ecology manual*, 3.3.1. Edited by A.D.L. Akkermans, J.D. van Elsas, and F.J. de Bruijn. Kluwer, Dordrecht. pp. 1–11.
- Döbereiner, J., and Day, J.M. 1976. First international symposium on nitrogen fixation. In *Proceedings of International Symposium on Nitrogen Fixation*. Edited by W.E. Newton and C.J. Nyman. Washington State University Press, Pullman, WA. pp. 518–538.
- Dreyfus, B.L., Elmerich, C., and Dommergues, Y.R. 1983. Free living *Rhizobium* strain able to grow on N₂ as the sole nitrogen source. *Appl. Environ. Microbiol.* **45**: 711–713.
- Eckert, B., Weber, O.B., Kirchoff, G., Halbritter, A., Stoffels, M., and Hartmann, A. 2001. *Azospirillum doebereineriae* sp. nov., a nitrogen-fixing bacterium associated with the C4-grass *Miscanthus*. *Int. J. Syst. Evol. Microbiol.* **51**: 17–26.
- Gogstad, G.O., and Krutnes, M. 1982. Measurement of protein in cell suspensions using the coomassie brilliant blue dye-binding assay. *Anal. Biochem.* **126**: 355–359.
- Grifoni, A., Bazzicalupo, M., Di Serio, C., Fancelli, S., and Fani, R. 1995. Identification of *Azospirillum* strains by restriction fragment length polymorphism of the 16S rDNA and of the histidine operon. *FEMS Microbiol. Lett.* **127**: 85–91.
- Gunarto, G., Adachi, K., and Senboku, T. 1999. Isolation and selection of indigenous *Azospirillum* spp. from a subtropical island, and effect of inoculation on growth of lowland rice under several levels of N application. *Biol. Fertil. Soils*, **28**: 129–135.
- Han, S.O., and New, P.B. 1998. Variation in nitrogen fixing ability among natural isolates of *Azospirillum*. *Microb. Ecol.* **36**: 193–201.
- Hartmann, A., and Zimmer, W. 1994. Physiology of *Azospirillum*. In *Azospirillum / plant associations*. Edited by Y. Okon. CRC Press Inc., Boca Raton, Fla. pp. 15–39.
- Hebbbar, P., Berge, O., Heulin, T., and Singh, S.P. 1991. Bacterial antagonists of sunflower (*Helianthus annuus* L.) fungal pathogens. *Plant Soil*, **133**: 131–140.
- Holguin, G., Patten, C.L., and Glick, B.R. 1999. Genetics and molecular biology of *Azospirillum*. *Biol. Fertil. Soils*, **29**: 10–23.
- Katupitiya, S., New, P.B., Elmerich, C., and Kennedy, I.R. 1995. Improved nitrogen fixation in 2,4-D treated wheat roots associated with *Azospirillum lipoferum*: studies of colonization using reporter genes. *Soil Biol. Biochem.* **27**: 447–452.
- Kennedy, I.R., Pereg-Gerk, L.L., Wood, C., Deaker, R., Gilchrist, K., and Katupitiya, S. 1997. Biological nitrogen fixation in non-leguminous field crops: facilitating the evolution of an effective association between *Azospirillum* and wheat. *Plant Soil*, **194**: 65–79.
- 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.
- Krieg, N.R., and Döbereiner, J. 1984. Genus *Azospirillum*. In *Bergey's manual of systematic bacteriology*. Edited by N.R. Krieg and J.G. Holt. Williams and Wilkins, Baltimore, Md. pp. 94–104.
- Ladha, J.K., So, R.B., and Watanabe, I. 1987. Composition of *Azospirillum* species associated with wetland rice plants grown in different soils. *Plant Soil*, **102**: 127–129.
- Lalande, R., Bissonnette, N., Coutlee, D., and Antoun, H. 1989. Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. *Plant Soil*, **115**: 7–11.
- Lindberg, T., and Granhall, U. 1984. Isolation and characterization of dinitrogen-fixing bacteria from the rhizosphere of temperate

- cereals and forage grasses. *Appl. Environ. Microbiol.* **48**: 683–689.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
- Magalhães, F.M.M., Baldani, J.I., Souto, S.M., Kuykendall, J.R., and Döbereiner, J. 1984. A new acid tolerant *Azospirillum* species. *An. Acad. Bras. Cienc.* **55**: 417–430.
- Michiels, K., Croes, C., and Vanderleyden, J. 1991. Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. *J. Gen. Microbiol.* **137**: 2241–2246.
- Miles, A.A., and Misra, S.S. 1938. The estimation of the bactericidal power of blood. *J. Hyg. (London)*, **38**: 732–749.
- Miller, J.H. 1972. Assay of β -galactosidase. In *Experiments of molecular genetics*. Cold Spring Harbour Laboratory Press, New York. pp. 352–355.
- Oh, K.H., Seong, C.S., Lee, S.W., Kwon, O.S., and Park, Y.S. 1999. Isolation of psychrotrophic *Azospirillum* sp., and characterization of its extracellular protease. *FEMS Microbiol. Lett.* **174**: 173–178.
- Okon, Y., and Hadar, Y. 1987. Microbial inoculants as crop-yield enhancers. *CRC Crit. Rev. Biotechnol.* **6**: 61–85.
- Okon, Y., and Kapulnik, Y. 1986. Development and function of *Azospirillum*-inoculated roots. *Plant Soil*, **90**: 3–16.
- Okon, Y., and Labandera-Gonzalez, C.A. 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. *Soil Biol. Biochem.* **26**: 1591–1601.
- Pereg-Gerk, L., Paquelin, A., Gounon, P., Kennedy, I.R., and Elmerich, C. 1998. A transcriptional regulator of the LuxR-UhpA family, FlcA, controls flocculation and wheat root surface colonization by *A. brasilense* Sp7. *Mol. Plant-Microbe Interact.* **11**: 177–187.
- Pereg-Gerk, L., Gilchrist, K., and Kennedy, I.R. 2000. Mutants with enhanced nitrogenase activity in hydroponic *Azospirillum brasilense*-wheat associations. *Appl. Environ. Microbiol.* **66**: 2175–2184.
- Pishchik, V.N., Mokrousov, I.V., Lazarev, A.M., Vorobyev, N.I., Narvskaya, O.V., Chernyaeva, I.I., Kozhemyakov, A.P., and Koval, G.N. 1998. Biological properties of some nitrogen-fixing associative enterobacteria. *Plant Soil*, **202**: 49–59.
- 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 associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). *Int. J. Syst. Bacteriol.* **37**: 43–51.
- Rodríguez Cáceres, E.A. 1982. Improved medium for isolation of *Azospirillum* spp. *Appl. Environ. Microbiol.* **44**: 990–991.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Sarig, S., Kapulnik, Y., and Okon, Y. 1986. Effect of *Azospirillum* inoculation on nitrogen fixation and growth of several winter legumes. *Plant Soil*, **90**: 335–342.
- Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology*, **1**: 784–791.
- Steenhoudt, O., and Vanderleyden, J. 2000. *Azospirillum*, a free-living nitrogen-fixing bacteria closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol. Rev.* **24**: 487–506.
- Tarrand, J.J., Krieg, N.R., and Döbereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions 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.
- Tyler, M.E., Milam, J.R., Smith, R.L., Schank, S.C., and Zuberer, D.A. 1979. Isolation of *Azospirillum* from diverse geographic regions. *Can. J. Microbiol.* **25**: 693–697.
- Vande Broek, A., Michiels, J., Van Gool, A., and Vanderleyden, J. 1993. Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression of the bacteria *nifH* gene during association. *Mol. Plant-Microbe Interact.* **6**: 592–600.
- Wong, P.P., Stenberg, M.E., and Edgar, L. 1980. Characterization of a bacterium of the genus *Azospirillum* from cellulolytic nitrogen fixing mixed cultures. *Can. J. Microbiol.* **26**: 291–296.
- Zeman, A.M.M., Tchan, Y.T., Elmerich, C., and Kennedy, I.R. 1992. Nitrogenase active wheat-root *para*-nodules formed by 2,4-dichlorophenoxyacetic acid (2,4-D) *Azospirillum*. *Res. Microbiol.* **143**: 847–855.