Development of molecular tools for the identification of free nitrogen-fixing bacteria: a contribution to the disclosure of soil microbial biodiversity

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Abstract

The preservation of microbial biodiversity offers indisputable benefits, both from an environmental and an economic point of view, due to the essential role of micro-organisms in the environment, and to their huge biotechnological potentiality.
Free nitrogen fixing bacteria (FNFB) represent a group of micro-organisms broadly dispersed in soil, water and sediments, which give a remarkable contribution to nitrogen fixation in the biosphere and could be usefully exploited as biological fertilisers and for other biotechnological applications. With the aim of providing a new, powerful tool for the study of these micro-organisms isolated from soil, we developed a method based on the 16S rDNA Amplified Ribosomal DNA Restriction Analysis (ARDRA) which was successfully used in order to identify 59 isolates from soils of central Italy.

Key words: ARDRA, *Azotobacteraceae*, Biodiversity, Free nitrogen fixing bacteria, Nitrogen fixation.

Riassunto

Sviluppo di metodi molecolari per l'identificazione di azotofissatori liberi come contributo allo studio della biodiversità microbica del suolo. La conservazione della biodiversità microbica offre indiscutibili benefici, sia dal punto di vista economico che ambientale, per il ruolo chiave che i microrganismi hanno nell'ambiente e per le loro enormi potenzialità biotecnologiche.
Gli azotofissatori liberi rappresentano un gruppo di microrganismi ampiamente diffusi nel suolo, nelle acque e nei sedimenti, con un ruolo fondamentale nella fissazione dell'azoto molecolare nella biosfera. Da ciò deriva l'interesse ad un loro potenziale impiego come fertilizzanti biologici, cui si accompagna anche l'interesse nei confronti di altre attività con possibili sviluppi in applicazioni biotecnologiche.
Al fine di fornire un nuovo, efficace strumento per lo studio di tali microrganismi isolati dal suolo, è stato messo a punto un metodo basato sull'analisi di restrizione del 16S rDNA amplificato via PCR (ARDRA), che è stato successivamente utilizzato per l'identificazione di 59 colture isolate da suoli del centro Italia.

Parole chiave: ARDRA, *Azotobacteraceae*, Azotofissatori liberi, Azotofissazione, Biodiversità.

Introduction

The microbial group of free nitrogen fixing bacteria (FNFB) includes several genera, as *Azotobacter*, *Azomonas*, *Azospirillum*, *Beierinckia* broadly dispersed in soil, water and sediments. These micro-organisms play a remarkable role in nitrogen fixation, increasing the content of fixed nitrogen in soil (Pandey *et al.*, 1998), and secrete stimulating substances, like gibberellins and indol-3-acetic acid (Russel, 1982). Due to these biochemical activities, FNFB have been employed in soil as biological fertilisers with the aim of reducing the input of chemicals in the natural environment, or in aquaculture systems and in vermicompost production (Kumar & Singh, 2001) with the aim of solubilising phosphates (Garg *et al.*, 2001). These evidences justify the interest recently arisen on the development of analytical tools suitable for a rapid and reliable identification of FNFB and for the study of these micro-organisms in soil microbial communities.

Materials and methods

Microorganisms

The following reference strains were used: *Azotobacter vinelandii* (DSM576, DSM2289, DSM2290, DSM87), *A. chroococcum* (DSM2286, DSM377), *A. armeniacus* DSM2284, *Azorhizophilus paspali* DSM2283, *A. beijerinkii* DSM378, *Azomonas macrocytogenes* DSM721, *A. agilis* DSM375, *A. insignis* DSM1845, *Azospirillum brasilense* DSM1690, *A. lipoferum* DSM1691, *A. amazonense* DSM2787, A. halopraeferens* DSM 3675, *Beijerickia indica* DSM 1715, *B. mobilis* DSM 2326, *B. fluminensis* DSM2327, *B. derxii* DSM2329 obtained from Deutsche Sammlung von Mikrorganism und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The strains *Agrobacterium tumefaciens* AT, *A. rhizogenes* AR, *A. radiobacter* DCBA10, *Sinorhizobium meliloti* DCBA8, *Rhizobium leguminosarum* DCBA11, *Pseudomonas corrugata* PC,
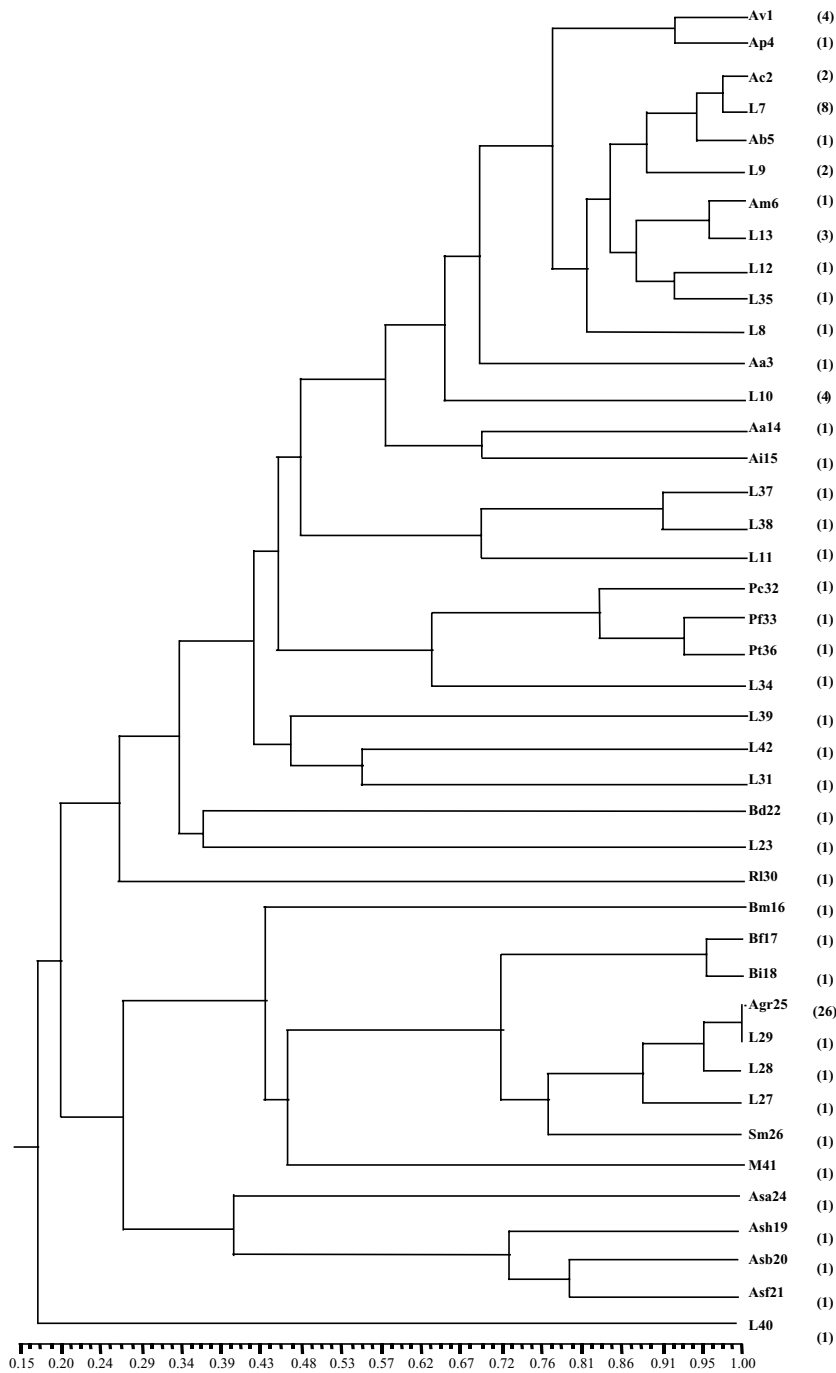
Av1	(4)	
Ap4	(1)	
Ac2	(2)	
L7	(8)	
Ab5	(1)	
L9	(2)	
Am6	(1)	
L13	(3)	
L12	(1)	
L35	(1)	
L8	(1)	
Aa3	(1)	
L10	(4)	
Aa14	(1)	
Ai15	(1)	
L37	(1)	
L38	(1)	
L11	(1)	
Pc32	(1)	
Pf33	(1)	
Pt36	(1)	
L34	(1)	
L39	(1)	
L42	(1)	
L31	(1)	
Bd22	(1)	
L23	(1)	
Rl30	(1)	
Bm16	(1)	
Bf17	(1)	
Bi18	(1)	
Agr25	(26)	
L29	(1)	
L28	(1)	
L27	(1)	
Sm26	(1)	
M41	(1)	
Asa24	(1)	
Ash19	(1)	
Asb20	(1)	
Asf21	(1)	
L40	(1)	

0.15 0.20 0.24 0.29 0.34 0.39 0.43 0.48 0.53 0.57 0.62 0.67 0.72 0.76 0.81 0.86 0.91 0.95 1.00

Fig 1 - Cluster analysis of total ribotypes using Dice coefficent of similarity. Numbers in brackets show the number of strains represented by each ribotype

P. fluorescens DCBA 14, *P. tolasii* DCBA4 were kindly provided by the Department of Chemistry and Agricultural Biotechnologies, University of Pisa (Italy). Strains were cultured overnight on Tryptic Soy Agar (TSA) or broth (TSB) (Oxoid) at 30°C and stored at –80°C in TSB containing 50 % (v/v) glycerol.

PREPARATION OF CRUDE CELL EXTRACT AND 16S RDNA AMPLIFICATION

DNA extraction and PCR reactions were performed as described by Aquilanti *et al*. (submitted).

DNA restrictions were performed with the five endonucleases *Rsa*I, *Hha*I, *Hpa*II, *Alu*I, *FnuD*II, as described by Aquilanti *et al.* (submitted).

Restriction patterns were converted into a binary matrix (Aquilanti *et al.*, submitted). The correlation matrix of the restriction patterns of each sample was performed using the DICE coefficient. The analysis was performed by using NTSYS-1.8 (Rohlf, 1993).

Results

The combination of profiles obtained from the references strains with the five restriction enzymes listed, led to define onto the 23 different species tested 21 unique species-specific ribotypes out of 22 totally obtained. Among the reference species tested, 19 were FNFB. Eight unique ribotypes were defined for all species within genera *Azotobacter* and *Azomonas* (Av1, Ac2, Aa3, Ap4, Ab5, Am6, Aa14) and seven unique ribotypes were defined for species belonging to genera *Azospirillum* (Ash19, Asb20, Asf21), and *Beijerinckia* (Bm16, Bf17, Bi18, Bd22) (Tab. 1).

Next, the ARDRA method was used to identify the 59 soil isolates of FNFB-like organisms previously screened. These isolates revealed to be distributed through out 11 different patterns or ribotypes (clusters). Some of them are represented by a single strain but others include several ones, as shown in Fig.1. A DICE similarity coefficient of 0.96 allowed differentiation at the species level among reference strains. Even though, DICE similarity coefficients of 0.75 or 0.80 are commonly used for microbial species identification, in this study we needed to fix an higher threshold, to obtain a discrimination at the species level between *A. chroococcum* (ribotype 2-Ab) and *A. beijerinckii* (ribotype 5-Ab), *A. vinelandii* (ribotype 1-Ab) and *A. paspali* (ribotype 4-Ab), and finally *Beijerinckia fluminensis* (ribotype 17-Bf) and *B. indica* (ribotype 18-Bi) (Fig 1).

As the identification of soil isolates at the species level is concerned, each strain was correlated to the most closely related reference culture (with a similarity coefficent higher than 80%).

Discussion

The development of a novel ARDRA method for reliable identification of *Azotobacteraceae* at the species level, started from planning of how many and which enzymes had to be employed. The number of enzymes was decided on the basis of two pivotal considerations: a computer analysis of digestion sites of over 100 environmental strains in the ribosomal database showed that the median sequence difference detected by the use of four tetrameric restriction endonucleases was 97,4% (Moyer *et al.*, 1996). Since organisms sharing less than 97,5% of 16S rRNA sequence are not recognised as members of the same species (Stackebrandt & Goebel, 1994), at least four enzymes result to be necessary to resolve the 16S rRNA gene of different species, although the use of additional endonucleases might allow a higher degree of discrimination. For this reason we decided to use five enzymes. The similarity dendrogram obtained from the cluster analysis confirmed, for reference strains, the phylogenetic relationships based on rRNA cistrons analysis proposed by De Smedth *et al.* (1980). As concerns environmental isolates, sixteen isolates out of 59 were clearly assigned into the FNFB group, being identified as members of the two species *A. chroococcum* and *A. macrocytogenes*.

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Tab. 1 - Results of ARDRA analysis on the Collection cultures used as a reference and on the 59 soil isolates. Aplotypes obtained from each restriction enzyme (column n. 1-5) and final ribotype (column n. 6) are shown

Strains	Aplotype					Ribotype
	Rsa I	*Hha* I	*Hpa* II	*Alu* I	*FnuD* II	
DSM 576, DSM 2289, DSM 2290, DSM 87	a	a1	a2	a3	a4	Av1
DSM 377, DSM 2286	a	b1	b2	a3	b4	Ac2
DSM 2284	a	c1	c2	b3	c4	Aa3
DSM 2283	a	d1	d2	a3	a4	Ap4
DSM 378	a	e1	e2	a3	b4	Ab5
DSM 721	a	f1	f2	a3	d4	Am6
BB4, BB3, AX8, AZ8, AX16, BB8, AZ2, BB6	a	b1	b2	a3	a4	L7
CC8	a	b1	b2	a3	t4	L8
AY14, AY7	a	b1	b2	b3	a4	L9
BG10, BG13, BG14, BG22	a	f1	b2	y3	l4	L10
BH23	a	b1	ad2	j3	u4	L11
BZ55, BN9	a	f1	b2	w3	a4	L12
BN16	a	f1	b2	a3	a4	L35
BG18, BG8, BP11	a	f1	b2	a3	d4	L13
DSM 375	b	g1	g2	c3	d4	Aa14
DSM 1845	c	h1	h2	c3	e4	Ai15
DSM 2326	d	l1	i2	d3	f4	Bm16
DSM 2327	e	l1	l2	e3	f4	Bf17
DSM 1715	e	n1	n2	e3	f4	Bi18
DSM 3675	e	p1	p2	h3	l4	Ash19
DSM 1690	e	o1	q2	l3	l4	Asb20
DSM 1691	e	r1	q2	l3	m4	Asf21
DSM 2329	f	m1	m2	f3	g4	Bd22
CH30	f	v1	y2	t3	t4	L23
DSM 2787	g	o1	o2	g3	h4	Asa24
AR, AT, DCBA 10 , BH27, BL22, BH31, BD2, BH34, BM33, BH29, BM23, BM28, BO27, BO7, BO41, CA28, BV62, BO18, BQ7, BQ32, BQ26, BQ15, BQ6, BY25, BT1, BU3	h	s1	r2	m3	n4	Ag25
DCBA 8	h	s1	s2	n3	o4	Sm26
CH10	h	w1	aa2	m3	t4	L27
BD2	h	w1	aa2	m3	t4	L27
BL46	h	s1	ae2	m3	n4	L28
CC103	h	s1	r2	m3	n4	L29
DCBA 11	i	t1	t2	o3	p4	Rl30
CA24	i	ab1	b2	x3	z4	L31
BY3	i	ab1	b2	x3	z4	L31
PC	l	u1	u2	p3	l4	Pc32
DCBA 14	l	u1	u2	q3	q4	Pf33
CD20	l	y1	ab2	r3	t4	L34
DCBA 4	m	u1	v2	q3	q4	Pt36
BH15	o	x1	ad2	j3	u4	L37
BH16	o	x1	ad2	a3	u4	L38
CH13	p	j1	w2	u3	t4	L39
CH4	q	k1	x2	v3	t4	L40
M1	r	w1	ac2	z3	t4	L41
BF15	s	aa1	b2	k3	v4	L42