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Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples

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Abstract

Due to the role of azotobacteria in nitrogen fixation and to their potential biotechnological applications, there is some challenge in developing an effective strategy for the selective isolation of these micro-organisms from soil. One hundred ninety-six Gram-negative strains were isolated from 35 soils sampled in central Italy, by using and comparing three different methods. The screening of soil samples by means of soil paste–plate method combined with isolation on mannitol-agar proved to be the best strategy in terms of reliability and selectivity. Moreover, preliminary recognition of free-living nitrogen-fixing isolates on differential LG medium revealed to be extremely accurate, since the majority of the isolates with *Azotobacter*-like morphology on such a medium were presumptively identified as members of the family Azotobacteraceae, by means of amplified ribosomal DNA restriction analysis.

Keywords: *Azotobacter*; Azotobacteraceae; Free-living nitrogen-fixing bacteria; ARDRA

1. Introduction

Nitrogen fixation can be considered as one of the most interesting microbial activity as it makes the recycling of nitrogen on earth possible and gives a fundamental contribution to nitrogen homeostasis in the biosphere. Among the free-living nitrogen-fixing bacteria those belonging to genus *Azotobacter* play a remarkable role, being broadly dispersed in different environments, such as soil, water and sediments (Palleroni, 1984). In fact, field trials have demonstrated that under certain environmental conditions, inoculation with *Azotobacter* has beneficial effects on plant yields (Jackson et al., 1964; Rovira, 1965; Denarié and Blanchere, 1966), due to the increase of fixed nitrogen content in soil (Gouri and Jagasnnatathan, 1995; Maltseva et al., 1995; Mrkovacki et al., 1996; Zahir and Arshad, 1996; Zahir et al., 1996; Pandey et al., 1998), and to the microbial secretion of stimulating hormones, like gibberellins, auxins and cytokinins (Mishustin and Shilnikova, 1971; Azcon and Barea, 1975; Martinez Toledo et al., 1989; Salmeron et al., 1990; Gonzales-Lopez et al., 1991). Several authors have shown the beneficial effects of *Azotobacter chroococcum* on vegetative growth and yields of maize (Mishra et al., 1995; Pandey et al., 1998; Radwan, 1998), as well as the positive effect of inoculation with this bacterium on wheat (Elshanshoury, 1995; Pati et al., 1995; Fares, 1997). Recently, it has been also shown that strains of *Azotobacter* could be usefully employed both in aquaculture systems (Garg et al., 2001) and in vermicompost production (Kumar and Singh, 2001), due to their ability of fixing nitrogen and solubilizing phosphates. An additional reason which justifies the interest on these micro-organisms is that the species *Azotobacter vinelandii* and *A. chroococcum* produce exopolysaccharides with high potential value related to their wide range of commercial applications (De la Vega et al., 1991; Peciña and Paneque, 1994; Clementi, 1997).

The present study was aimed to design a simple and efficient strategy for the isolation of *Azotobacter* from soil. Indeed, most of the methods available in literature show a significant number of inconsistencies in the composition of media, possibly due to the numerous modifications proposed by different authors over the past years and the varied published sources from which the methods are

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derived (Knowles, 1982). The other target of this work was the development of a method for: (i) screening and preliminary recognition of cultures belonging to genus *Azotobacter*; (ii) first identification of isolates, on the basis of amplified ribosomal DNA restriction analysis (ARDRA). This latter goal is particularly important because the unequivocal characterisation of *Azotobacter* at the species level is not so easily achieved through classical phenotypic methods, mainly due to considerable variations in many phenotypic traits (Jayarao et al., 1992). In doing so, we also intended to provide a useful device for a further investigation of the microbial diversity and the environmental distribution of *Azotobacter* in soil.

2. Materials and methods

2.1. Micro-organisms

Azotobacter vinelandii DSM576, *Azotobacter vinelandii* DSM2289, *A. vinelandii* DSM2290, *A. vinelandii* DSM87, *A. chroococcum* DSM2286, *A. chroococcum* DSM377, *Azotobacter armeniacus* DSM2284, *Azotobacter beijerinkii* DSM378, *Azorhizophilus paspali* DSM2283, *Azomonas macrocytogenes* DSM721, *Azomonas agilis* DSM375, *Azomonas insignis* DSM1845, *Azospirillum brasilense* DSM1690, *Azospirillum lipoferum* DSM1691, *Azospirillum amazonense* DSM2787, *Azospirillum halopraeferens* DSM3675, *Beijerinckia indica* DSM1715, *Beijerinckia mobilis* DSM2326, *Beijerickia fluminensis* DSM2327, *Beijerickia derxii* DSM2329, were obtained from Deutsche Sammlung von Mikrorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Agrobacterium tumefaciens* AT, *Agrobacterium rhizogenes* AR, *A. radiobacter* DCBA10, *Sinorhizobium meliloti* DCBA8, *Rhizobium leguminosarum viciae* DCBA11, *Pseudomonas corrugata* PC, *Pseudomonas fluorescens* DCBA14, and *Pseudomonas tolasii* DCBA4 were kindly provided by the Department of Chemistry and Agricultural Biotechnologies, University of Pisa (Italy). All strains were cultured on Tryptic Soy Agar (TSA) or broth (TSB) (Oxoid, Basingstoke, UK) overnight at 30 °C and stored frozen in 50% (v/v) glycerol in TSB at −80 °C.

The above listed reference strains were used as a control on all the different media employed.

2.2. Soil sampling

Soil samples were collected during Spring and Autumn 2000 in different areas of central Italy both from cultivated and uncultivated soils. Samples were withdrawn at a depth of 10–15 cm below the surface, collected into sterile vials as described by Kole and Altosaar (1988), sieved through a 4-mm-mesh sieve, and stored at field moisture content at 4 °C. Soil pH was measured according to Van Lierop (1981), analysing samples thoroughly suspended in distilled water 1:1 (v/v).

2.3. Isolation

Three different isolation strategies were used: (a) streaking of serial soil dilutions on plates containing Brown N-free medium (Brown et al., 1962; Knowles, 1982); (b) enrichment in Winogradsky solution for 7–14 days (Augier, 1956; Pochon and Tardieux, 1962) followed by streaking onto Brown-agar; (c) a combination of the soil paste (Becking, 1981) and the direct sowing of single soil grains (Pochon, 1954) methods realised as follows: about 30–50 g of each soil sample were accurately mixed with 20% (v/w) of sterile water and 1% (w/w) of mannitol. The soil paste, prepared in a porcelain mortar, was transferred and pressed inside a petri dish with a sterile spatula to obtain a smooth and levelled surface. After 3–7 days incubation at 27–30 °C, the soil paste–plates presenting growth of *Azotobacter* were revealed by the appearance of slimy, glistening colonies, turning brown with aging if produced by the species *A. chroococcum*. Subsequently, in order to carry out isolation, soil samples resulted positive for the presence of these free-living nitrogen-fixing bacteria were subjected to sowing of single grains on the N-free medium proposed by Pochon (1954), containing mannitol as C-source and solidified with purified agar instead of silica gel (Becking, 1981).

All the isolates were purified by streaking on TSA plates. Long-term storage of the purified isolates was at −80 °C in TSB (Oxoid) with 50% (w/v) glycerol. Short-term storage for further characterisation was on TSA plates at 4 °C.

2.4. Screening of isolates

To differentiate isolates between Gram-positive and Gram-negative, the KOH-test described by Ryu (1938) was used. Cultures giving uncertain results were subjected to Gram staining. Isolates were then screened for growth ability, colony morphology, pigments production and acidification activity on N-free LG medium (Turner and Gibson, 1980) containing sucrose as sole carbon source and blue of bromothymole as pH indicator. Production of acid, indicated by a change in colour of the medium from blue or dark green to a definite yellow, was checked and recorded after 2, 5, or 7 days incubation, as well as pigments production and colony morphology.

2.5. Total DNA extraction

For molecular analysis, bacteria were grown for 2–3 days on TSA. Crude template DNA was prepared as following: two or three colonies were suspended in 500 μl of sterilised ultrapure water (Millipore-Q, Bedford, USA), collected by centrifugation, resuspended in 100 μl of water, added with 100 μl of 10 mM Tris–HCl pH 8.2 and vortexed. Finally, 13 μl of 1 mg/ml proteinase K (Sigma, St Louis, USA) were added. After incubation at 55 °C for

2 h, cells undergone to lysis were pelleted by centrifugation and 2 μl of the supernatant fluid were used for the PCR amplification.

2.6. ARDRA

The amplification of the 16S rRNA gene amplification via PCR was performed in a 50 μl reaction mixture containing: 1 U of Taq DNA polymerase (Amersham Biosciences, Uppsala, Sweden), 10 mM Tris–HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 μM of dNTPs, and 2 μM of each universal primer 27f and 1495r (Weisburg et al., 1991) purchased from MWGH-Biotech (Ebersberg, Germany). These primers, designed on the basis of conserved sequences of *Eubacteria*, are located at the extreme 5′ and 3′ of the 16S rRNA gene, respectively, allowing an approximately 1500-bp DNA fragment to be amplified. Methods described by Nuzzi (1997) were employed for the PCR amplification. Briefly, after incubation at 95 °C for 4 min, the reaction mixture was cycled through the following temperature profile: initial denaturation at 95 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, each. The PCR session was terminated at 72 °C for 15 min, following by cooling at 4 °C. DNA amplification was checked by electrophoresis of 8 μl of each PCR product in a 1.5% (w/v) agarose gel, in TBE buffer (0.09 M Tris base, 0.09 M sodium borate, 2.5 mM EDTA, pH 8.3) for 1 h at 3.2 V/cm. A 12.5–μl-aliquot of the PCR product was digested in a final volume of 25 μl for 8 h at 37 °C with 5 U of restriction enzyme (*Rsa* I or *Hha* I) according to the manufacturer's specifications (Roche, Basel, Switzerland). DNA digests were separated by 2.5% (w/v) agarose gel electrophoresis in TBE buffer for 4 h at 3.2 V/cm. Gels were stained in ethidium bromide for 15 min and thereafter washed for 5 min. DNA fragments were visualised at 312 nm with a UV-transilluminator ImageMaster VDS (Amersham Biosciences). Gels' electronic images captured with LISCAP software v.1.0 (Amersham Biosciences). Molecular identification of the soil isolates was realised comparing their restriction profiles with those of the above listed reference strains.

3. Results

3.1. Soil sampling

The sampling strategy described in this work was chosen taking into account the different parameters influencing the presence of azotobacteria in soil. Since distribution of *Azotobacter* in the rhizosphere is not dependent on the type of plant (Kole and Altosaar, 1988) soil samples were indifferently collected from the rhizosphere of corn, wheat, and common lawn grasses. Moreover, as the use of fertilizers in soils tend to favour the development of micro-organisms unable to fix nitrogen, the majority of samples were taken from uncultivated soils. On the other hand, since the diffusion of azotobacteria in soil is encouraged by richness in organic matter (Jensen, 1965; Mishustin and Shilnikova, 1971; Thomson and Skerman, 1979; Becking, 1981; Gordon, 1981) and in phosphates, which are particularly important for the *Azotobacter* metabolism (Tsai et al., 1979), the remaining samples were withdrawn from cultivated soils (Table 1). Distribution of *Azotobacter* in soil is also greatly influenced by the pH value. In fact, their populations reach the highest levels in soils with pH above 6.5, whereas they rarely occur when pH is below 6.0 (Jensen, 1965). All the soil samples collected showed pH values within the range 6.5–9.0 and were therefore used for *Azotobacter* isolation (Table 1).

3.2. Isolation strategies

The three methods utilised in the present work were described by different authors as feasible for *Azotobacter* isolation and their discrimination from other free-living nitrogen-fixing soil bacteria. In order to assess the reliability of these methods, they were preliminary tested on the reference strains, and subsequently utilised for the isolation of *Azotobacter* from 35 soil samples.

Method 'a' allowed the direct isolation of *Azotobacter*-like colonies on selective Brown medium from 16 out of 35 soil samples utilised. All members of genus *Azotobacter* produced slimy, glistening, smooth, whitish, weakly convex, 2–10-mm in diameter colonies. However, slight differences in size and sliminess of colonies were observed among different species. *A. vinelandii* formed circular, 2–3 mm in diameter, weakly slimy colonies (Fig. 1a), while only strain DSM87 produced a green fluorescent undiffusible pigment. *A. armeniacus* formed irregular, large, very slimy colonies and did not reveal any pigment production. *A. chroococcum* produced regular, very small colonies which turn brown with age. *A. paspali* formed rough, matt colonies with irregular edges.

With the aim of assessing Brown medium selectivity, reference strains belonging to genera *Azomonas*, *Azospirillum*, *Beijerinckia*, *Agrobacterium* and *Rhizobium* were also seeded on this medium. It resulted that within genus *Azomonas*, *A. macrocytogenes* was characterised by smooth, deeply slimy, convex or domed and large (1–6 mm in diameter) colonies. Moreover, due to the release of a diffusible pigment, the whole plate turned to pink-purple after 3–5 days incubation. Growth of *A. agilis* and *A. insignis* was revealed by matt, smooth, medium-size (2–6 mm) colonies, while pigment production was detected. Members of genus *Azospirillum* were easily recognised by the production of a blue diffusible pigment. Colonies were small and glistening, but no slime production was observed. *Beijerinckia* produced large, non-pigmented and slimy colonies. Unexpectedly, members of genera *Agrobacterium* and *Rhizobium* grew on Brown medium,

Table 1
Soil samples utilised for the isolation of *Azotobacter*

N°	Sample	Sampling site	Soil texture	pH	Isolation method
1	AR	River bank	Silt	7.85	a (0)
2	AS	Lawn grasses	Clayey soil	7.10	a (0)
3	AT	Beech wood	Loam	7.45	a (0)
4	AU	Coast	Sandy soil	8.60	a (0)
5	AV	Coast	Sandy soil	8.60	a (3)
6	AX	Garden	Clayey soil	7.47	a (3)
7	AY	Garden	Clayey soil	7.47	a (5)
8	AZ	Lawn grasses	Clayey soil	7.10	a (15)
9	BA	Lawn grasses	Clayey soil	7.32	a (0)
10	BB	Lawn grasses	Clayey soil	7.45	a (4)
11	BC	Lawn grasses	Clayey soil	7.30	a (0)
12	BD	Lawn grasses	Clayey soil	7.55	b (1)
13	BF	Field	Clayey soil	7.82	a (2)
14	BG	Wheat cultured soil	Clayey soil	8.70	a (14)
15	BH	Wheat cultured soil	Clayey soil	7.75	a (10), b (1)
16	BK	Lawn grasses	Clayey soil	7.83	a (5), b (8)
17	BM	River bank	Silt	7.64	a (33)
18	BN	Birch wood	Clayey soil	6.50	a (4)
19	BO	Pit	Calcareous soil	8.90	a (10)
20	BP	Limestone pit	Calcareous soil	8.90	a (5)
21	BQ	Dump	Clayey soil	8.45	a (6), b (10)
22	BR	Dump	Clayey soil	8.10	a (2), b (2)
23	BS	Dump	Clayey soil	8.10	b (1)
24	BT	Lawn grasses	Clayey soil	8.35	b (1)
25	BU	Rural pathway borders	Calcareous soil	7.95	b (0)
26	BV	Urban street borders	Clayey soil	7.72	a (9)
27	BX	Urban street borders	Clayey soil	7.78	a (0)
28	BY	Urban street borders	Clayey soil	7.72	b (1)
29	BZ	Urban street borders	Clayey soil	7.83	b (0)
30	CF	Maize cultured soil	Clayey soil	8.00	c (8)
31	CG	Maize cultured soil	Clayey soil	8.30	c (16)
32	CH	Maize cultured soil	Clayey soil	8.00	c (8)
33	CI	Greenhouse	Loam	7.20	c (0)
34	CL	Garden	Clayey soil	7.15	c (0)
35	CM	Garden	Clayey soil	8.26	c (2)

Number of strains isolated for each soil sample is given in parentheses. Isolation methods are indicated as follows: a, streaking of serial soil dilutions on plates containing Brown N-free medium; b, enrichment in Winogradsky solution for 7–14 days followed by streaking onto Brown-agar; c, combination of the soil paste and the direct sowing of single soil grains methods.

as well, producing colonies 1–3 mm in diameter and clearly transparent, which were similar to those formed by members of the genus *Azotobacter*.

Method 'b' was tested on 10 soil samples. As expected, growth of *Azotobacter*, *Azomonas*, *Azospirillum* and *Beijerinkia* references strains on Winogradsky solution was revealed by an increase of turbidity and the appearance of a thin pellicle on the liquid surface. Moreover, growth of most *Azotobacter* was accompanied by the production of diffusible pigments. A fluorescent green pigment was produced by *A. vinelandii* DSM576, DSM2289, DSM87, whereas a purple and a yellowish pigment were produced by *A. vinelandii* DSM2290 and *A. paspali* DSM2283, respectively. *A. chroococcum* DSM2286 and DSM377 did not release diffusible pigments, but a characteristic brown ring was formed on the liquid surface after 5–6 days incubation.

Although, the results observed with the reference strains in Winogradsky solution were unambiguous, those obtained after inoculation with soil sample dilutions were not so easily understandable. Indeed, growth of different micro-organisms (e.g. aerobic and micro-aerophilic species in the nearby of the liquid surface) led to the production of a milky and creamy pellicle, browning with aging, and to a significant increase in turbidity which rendered the observation of diffusible pigments not possible (Fig. 1b). Consequently, due to the impossibility to individuate the positive tubes for *Azotobacter*, pellicles coming from all the tubes showing growth were streaked onto Brown medium. In this way, two objectives were contemporary pursued: the individuation of *Azotobacter*-like colonies and the achievement of pure cultures. As a result, 23 putative *Azotobacter* were isolated from eight out of the 10 soil samples screened.

According to method 'c', soil samples to be employed in the isolation step were selected by means of the soil paste–plate technique, thanks to the appearing of slimy

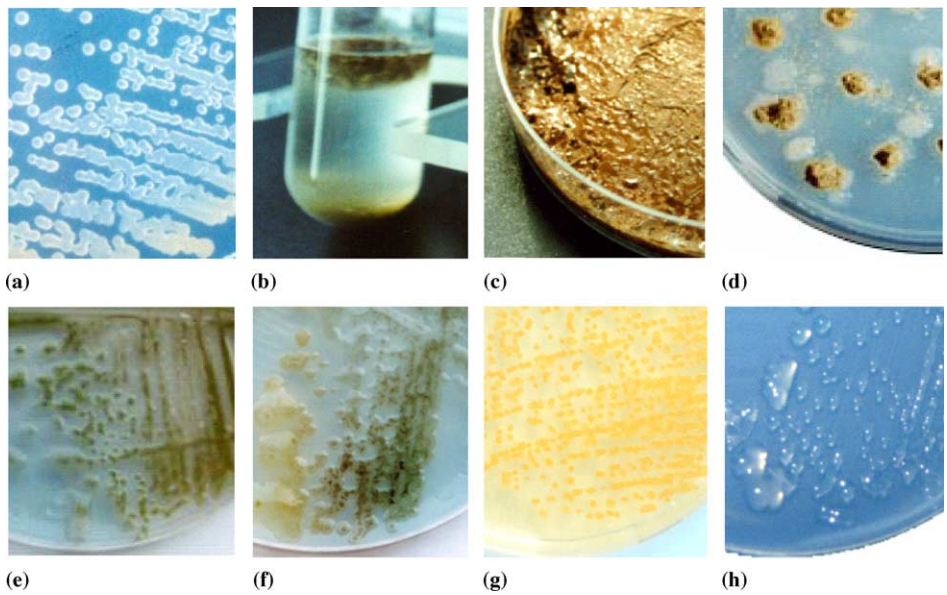

Fig. 1. Enrichment, isolation, and screening media for azotobacteria. (a) colonies of *Azotobacter vinelandii* DSM576 onto Brown-agar medium; (b) creamy and brown ring formed by micro-organisms grew in Winogradsky solution after inoculation with soil; (c) aqueous and translucent colonies formed by free nitrogen-fixing bacteria upon the smoothed soil paste–plate surface; (d) *Azotobacter*-like colonies grew around the single soil grains sewed onto the mannitol-agar medium; (e) *Azotobacter vinelandi* DSM2289 colonies onto differential LG agar medium; (f) *Azotobacter armeniacus* DSM2284 colonies onto differential LG agar medium; (g) *Azotobacter paspali* DSM2283 onto differential LG agar medium; (h) *Agrobacterium tumefaciens* AT onto differential LG agar medium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

and glistening colonies upon the smoothed soil paste surface (Fig. 1c). Four out of six soil samples screened were therefore selected and utilised for *Azotobacter* isolation onto mannitol medium, through the direct sow of single soil grains (Fig. 1d). Mannitol N-free substrate revealed to be particularly suitable for the presumptively recognition of *A. chroococcum*, since both reference strains tested formed up to 10 mm in diameter colonies, gradually turning to black with aging. As previously observed, also all the other reference strains tested were able to grow, producing small, glistening, and smooth colonies. The utilisation of this combined method led to the isolation of 35 *Azobacter*-like cultures.

3.3. Screening of the isolates

Once assessed that the 196 soil isolates were Gram-negative, these and the reference strains were screened onto the N-free LG medium, being clustered into 10 groups on the basis of their phenotypical traits (Table 2). Briefly, growth of *Azotobacter* reference strains was characterised by the production of glistening, and slimy colonies, due to the capability of these micro-organisms of metabolising sucrose (Turner and Gibson, 1980). Yellow-green pigments were produced by all the *Azotobacter* species tested (Fig. 1e–g), except for *A. chroococcum*, which produced a brown pigment. The other free-living nitrogen-fixing genera taken under study grew on LG medium as well, producing *Azotobacter*-like colonies. Among these, *Azomonas agilis*, whose ability to utilise sucrose as sole carbon source was not described so far (Tchan and New, 1984). Except for *Azomonas agilis* and *Azospirillum amazonense* all the nitrogen-fixing species tested caused an acidification of the medium, revealed by a defined change in colour.

LG medium selectivity was tested by evaluating its ability to support growth of *A. radiobacter*, *A. tumefaciens*, *A. rhizogenes*, *S. meliloti* and *R. leguminosarum viciae* reference strains. On such a medium, these strains produced colourless, translucent, aqueous colonies, easily distinguishable from the *Azotobacter*-like colonies. Neither acidification activity nor pigment production were observed (Fig. 1h).

Among the 196 isolates screened on LG medium, 49 were ascribed to groups III, IV, VI, VII, and VIII, being preliminary assigned to the family Azotobacteraceae, while the remaining ones were clustered in LG group X (Table 2).

3.4. Identification of the isolates by means of ARDRA

The enzymes utilised for ARDRA, namely *Rsa* I and *Hha* I, were chosen on the basis of the outcomes previously obtained by Nuzzi (1997).

Results obtained onto the reference strains indicated that *Rsa* I produced a characteristic five-band restriction profile for all the *Azotobacter* species analysed, plus *Azomonas macrocytogenes* (Fig. 2a). Indeed, for *Azomonas agilis* and *A. insignis*, two unique species-specific profiles were defined (Fig. 2a). Moreover, the utilisation of such an enzyme was sufficient to discriminate members of the family Azotobacteraceae both from bacteria belonging

Table 2
Screening of soil isolates and reference strains onto LG medium, based on: colony size and morphology, pigments production, and acid production showed by a change in colour of the medium from blue to a definite yellow

Group	Reference strains	Colony morfology	Pigmentation	Colour change	Soil isolates
I	*A. vinelandii* DSM2289, DSM2290	Large [a], slimy, glistening	Brown-green, purple	+	n.i.
II	*A. vinelandii* DSM576, DSM87	Small [b], glistening	Yellow-green	+	n.i.
III	*A. chrococcum* DSM2286, DSM377	Small, glistening	Brown	+	AX8,16;AZ2,8;BB3,4,6,8; CM8;CG3,10,13;CF13; CH4,7,10,11,13
IV	*A. paspali* DSM2283 *A. beijerinckii* DSM378 *B. mobilis* DSM2326 *B. fluminensis* DSM2327 *B. derxii* DSM2329 *B. indica* DSM1715 *A. brasilense* DSM1690 *A. lipoferum* DSM1691 *A. halopraeferens* DSM3675	Small, glistening	Yellow	+	AY7,14
V	*A. armeniacus* DSM2284	Large, slimy, glistening	Yellow	+	n.i.
VI	*A. macrocytogenes* DSM721	Large, slimy, convex	None	+	AY3;BG6,8,9,10,13,14,18, 22,23.1,23.2;BH23,27,31;BN9, 16,11,36;BP11,36;BZ55; CG1,15;CF2,4,5,7
VII	*A. insignis* DSM 1845	Small, matt	Yellow	+	BV67
VIII	*A. agilis* DSM 375	Medium-size [c], slimy	Brilliant green	–	BY3w
IX	*A. amazonense* DSM 2787	Small, matt	–	–	n.i.
X	*A. rhizogenes* AR *A. tumefaciens* AT *A. radiobacter* DCBA 10 *R. meliloti* DCBA11 *P. corrugata* PC *P. fluorescens* DCBA14 *P. tolasii* DCBA4	Medium-size, slimy, glistening	–	–	AV5,9,12;AY1,4;AX9;AZ1,3, 4,4.3,5,5.3,6,7,9,9.2,9.3,10, 17;BD4w,BF15,16;BG27,29, 31,34;BH3w,15,16,21,29,30, 33,34;BK3,4,6,45,46,11w,12w, 13w,14w,15w,18w,21w,22w; BM17,18,19,20,21,22,23,24, 25,26,27,28,29,30,31,32,33, 34,35,36,37,38,39,40,41, 42,43,44,45,46, 47,48,50;BN20; BP8,24,58;BQ12,14,27,28,39,41.1, 1w,2w,3w,5w,9w,12w,15w,26w, 27w,29w,32w;BO3,7,15,18,25, 27,33,40,41,69;BR5,8,3w,11w; BS8w;BT1w,3w,4w;BV1,4,5, 6,12,13,14,16;CF1,9, 11;CG2,4,5,6,7,8,9,11,12,14,16,17, 18;CH8,12,30;CM1

Number of each isolate is preceded by soil sample code. ni, no isolates obtained.
[a] Large, 5–10 mm in diameter.
[b] Small, 1–3 mm in diameter.
[c] Medium-size, 3–5 mm in diameter.

to the free-living N-fixing genera *Beijerinckia* and *Azospirillum* (Fig. 2b) and to the related genera *Agrobacterium*, *Rhizobium* and *Pseudomonas* (Fig. 2c). As concerns *Agrobacterium* and *Pseudomonas*, two genus-specific profiles were found (Fig. 2d). Restriction analysis with *Hha* I onto the reference strains allowed eight unique species-specific restriction profiles to be obtained within the family Azotobacteraceae (Fig. 2d).

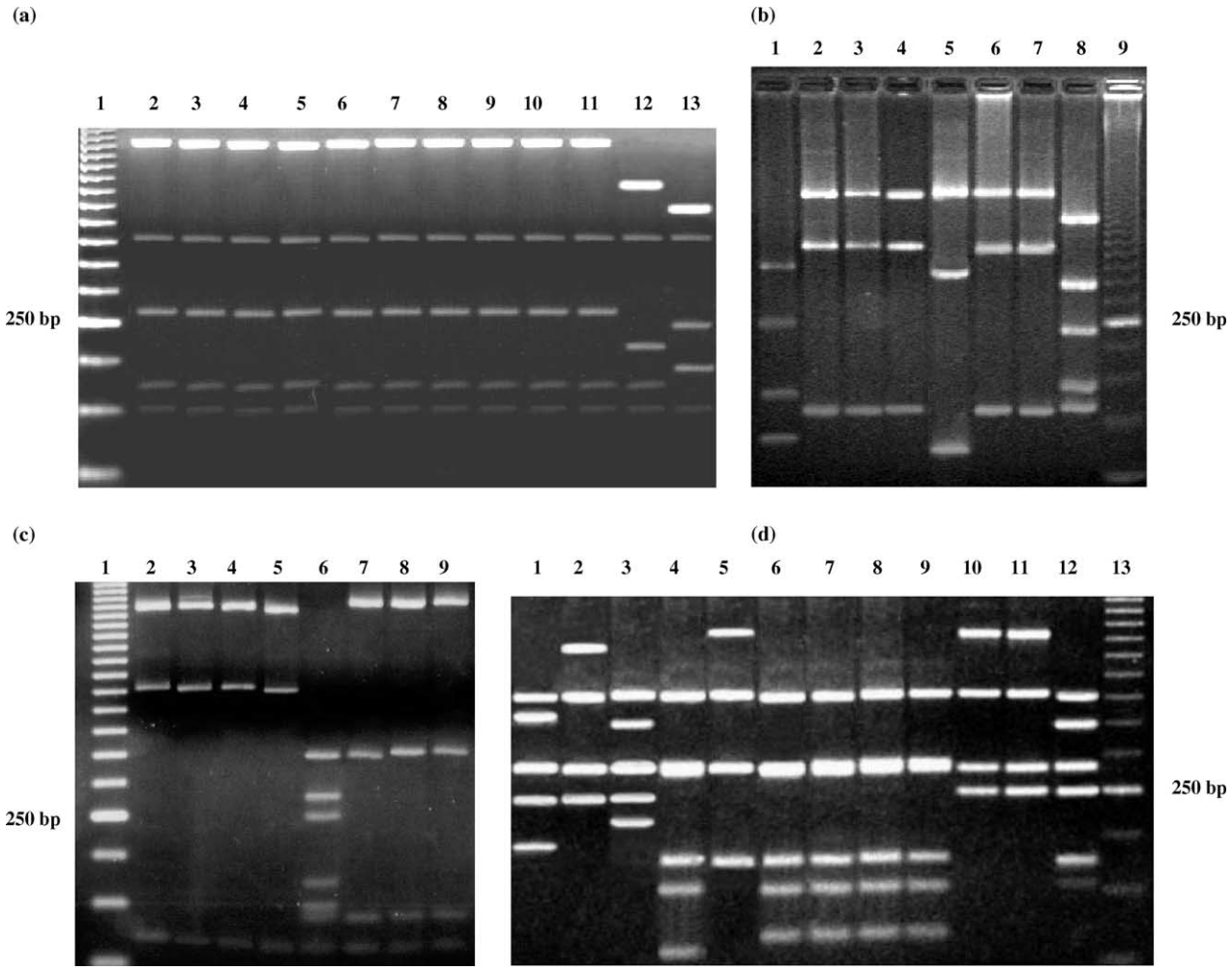

Fig. 2. Agarose gel electrophoresis of restriction fragments obtained from the digestion of 16S rDNA amplicons of reference strains. (a) ARDRA profiles of Azotobacteraceae references strains obtained with the enzyme *Rsa* I. Lane1-*Azotobacter vinelandii* DSM576, 2-*A. vinelandii* DSM87, 3-*A. vinelandii* DSM2289, 4-*A. vinelandii* DSM2290, 5-*A. chroococcum* DSM377, 6-*A. chroococcum* DSM2286, 7-*A. armeniacus* DSM2284, 8-*A. beijerinckii* DSM378, 9-*A. paspali* DSM2283, 10-*Azomonas agilis* DSM375, 11-*A. insignis* DSM1845, 12-*A. macrocytogenes* DSM721; (b) ARDRA profiles of the free-living nitrogen-fixing references strains, obtained with the enzyme *Rsa* I. Lane1-*B. derxii* DSM2329, 2-*B. indica* DSM2327, 3-*B. mobilis* DSM2326, 4-*B. fluminensis* DSM2327, 5-*Azospirillum amazonense* DSM2787, 6-*A. brasilense* DSM1690, 7-*A. halopreferens* DSM3675, 8-*Azotobacter vinelandii* DSM576, 9-50 base pair ladder; (c) ARDRA profiles of references strains unable to fix dinitrogen in a free-living form, obtained with the enzyme *Rsa* I. Lane 1-50 base pair ladder 2-*A. tumefaciens* AT, 3-*A. rhizogenes* AR, 4-*A. radiobacter* DCBA10, 5-*S. meliloti* DCBA8, 6-*R. leguminosarum viciae* DCBA11, 7-*P. corrugata* PC 8-*P. fluorescens* DCBA14, 9-*P. tolasii* DCBA4; (d) ARDRA profiles of Azotobacteraceae references strains obtained with the enzyme *Hha* I. Lane1-*Azotobacter vinelandii* DSM576, 2-*A. vinelandii* DSM87, 3-*A. vinelandii* DSM2289, 4-*A. vinelandii* DSM2290, 5-*A. chroococcum* DSM377, 6-*A. chroococcum* DSM2286, 7-*A. armeniacus* DSM2284, 8-*A. beijerinckii* DSM378, 9-*A. paspali* DSM2283, 10-*Azomonas agilis* DSM1845, 11-*A. insignis* DSM375, 12-*A. macrocytogenes* DSM721, 13–50 base pair ladder.

Rsa I analysis onto the soil isolates revealed that only 40 cultures out of the 49 preliminary assigned to the family Azotobacteraceae (Table 2) could be effectively ascribed to this microbial family, showing a characteristic *Rsa* I *Azotobacter* or *Azomonas* restriction pattern. Indeed, nine out of 49 *Azotobacter*-like isolates (BG22,23.1,23.2; BN11,36; BZ55; AZ2,8; CM8) (Table 2) were misidentified onto LG, showing the characteristic *Rsa* I *Agrobacterium*/*Rhizobium* restriction pattern. On the contrary, the 147 isolates previously assigned to group X (Table 2), were confirmed as members of genera *Agrobacterium*/*Rhizobium*, being discarded for further *Hha* I restriction analysis.

The 40 isolates selected on the basis of the outcomes obtained with *Rsa* I were therefore subjected to *Hha* I restriction analysis, in order to succeed a more defined assignation into species. According to the results obtained, 15 isolates were ascribed to the species *A. chroococcum*, one to the species *A. insignis*, one to the species *A. agilis* and 20 to the species *A. macrocytogenes*.

4. Discussion

This research work firstly aimed to compare three different methods reported in literature for the isolation of

free N-fixing bacteria from soil samples, in order to individuate the most effective one. In second instance, it aimed to verify whether LG medium, described up to now as a selective substrate for the isolation of *Azotobacter* can be successfully employed to screen soil isolates for a presumptive recognition of micro-organisms belonging to the genus of interest.

The three methods compared, although described as selective for *Azotobacter*, led to the isolation of a large number of soil bacteria unable to fix nitrogen. This lack of selectivity, possibly due to the ability of such isolates to act as oligonitrophiles (Knowles, 1982), represents one of the major problems in isolating *Azotobacter*. In particular, contrarily to what reported by Knowles (1982) the colony morphology of *Azotobacter* on Brown medium is not clearly recognisable. Similarly, the enrichment on Winogradsky solution proved not to be selective enough. In fact, the pellicle or ring on the liquid surface of the enrichment medium was formed not only by aerobic N-fixing micro-organisms, but also by bacteria unable to fix nitrogen. This fact was probably due to the high amount of soil inoculated in the enrichment solution. It is known that the validity of an enrichment method largely depends on the ease with which the micro-organisms of interest are able to develop from small inocula (Knowles, 1982). Under the experimental conditions described, more competitive micro-organisms other than N-fixing bacteria, may prevail because of the presence of a certain content of fixed nitrogen and non-selective carbon sources.

On the contrary, the combination of paste–plate and soil grains sowing methods seems to be more efficient in terms of rapidity and feasibility. In fact, the soil paste–plate technique revealed to be extremely useful for a preliminary screening of soil samples allowing those containing N-fixing bacteria to be recognised and subsequently used for isolation. Moreover, the following streacking and incubation on mannitol medium permit a rapid individuation of slimy and glistening *Azotobacter*-like colonies.

Results obtained in this work also suggested that the utilisation of LG medium has to be reconsidered. Indeed, this medium revealed to be differential more than selective, allowing free-living diazotrophes to be preliminarily recognised on the basis of morphology, consistency and pigmentation of the colonies. Moreover, the presence of a pH indicator permitted the individuation of the *Azotobacter* acidifying strains. LG usefulness in the individuation of Azotobacteraceae was confirmed by ARDRA. Indeed, molecular characterization of the isolates showed that only nine out of 43 were misidentified on LG.

In conclusion, our results showed that the most reliable strategy for the isolation and preliminary identification of *Azotobacter* is given by the combination of paste–plate and soil grains sowing methods followed by screening on LG.

As concerns assignation into species by means of ARDRA, the utilisation of universal primers complementary to well-conserved regions in the prokaryote genome, led to the amplification of the 16S rRNA gene of isolates belonging both to genus *Azotobacter* and to other related genera. This allowed to compare and better evaluate selectivity of the isolation strategies tested, extending preliminary identification to the major number of species possible.

The use of the two restriction enzymes *Rsa* I and *Hha* I revealed to be suitable for a preliminary screening of the isolates, allowing a clear differentiation between all the Azotobacteraceae species tested, through the production of genus- and species-specific profiles.

Moreover, the possibility of atypical profiles was not taken into account on the basis of the high intra-specific homogeneity of ARDRA profiles observed for the references strains belonging to the two species *A. vinelandii* and *A. chrococcum*. No atypical *Azotobacter* or *Azomonas* profiles were observed among isolates and, as a consequence, restriction analysis with additional enzymes was not applied.

Though the small number of samples analysed in this study is too scarce in order to give information on the ecology of *Azotobacter* in soil examined, some considerations can be attempted. *A. chroococcum* was the prevalent species among isolates identified as members of the family Azotobacteraceae. The closely related species *A. insignis* and *A. agilis* were also isolated, but only occasionally, partially due to their inability to utilise mannitol as sole carbon source, and to a predilection of these bacteria for water environments (Becking, 1981).

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