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Immunological Comparison between *Arthrobacter* Isolates and Bacteria Living in *Azolla filiculoides* Lam.

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Abstract

Antisera were prepared against two *Arthrobacter* strains isolated from *Azolla filiculoides* and the type strain *Arthrobacter globiformis* ATCC 8010. The antisera confirmed that the bacteria associated with *Azolla filiculoides-Anabaena* belong to the genus *Arthrobacter*. Differences in the immunological reactions among the different species *Arthrobacter* were noted, indicating the presence of common antigens in the two forms, coccus and rod. This contributes to the knowledge of the pleomorphism of *Arthrobacter*. The presence of gram-positive *Arthrobacter* in the *Azolla-Anabaena* association was also confirmed by immunogold labeling of the bacteria in the leaf cavities of *Azolla filiculoides*.

Keywords: *Azolla*, *Arthrobacter*, antibodies, immunogold labeling

1. Introduction

Bacteria belonging to different species of the genus *Arthrobacter* Conn and Dimmick, are present in the leaf cavities of the fern *Azolla* Lam. (Wallace and Gates, 1986; Forni et al., 1989), as well as in the sporocarps of *A. filiculoides* Lam. (Forni et al., 1990). This genus is known for its Gram variability, which results in a number of gram-negative cells in a gram-positive culture and for the pleomorphism due to a rod-coccus cycle, in which rods appear during the log phase and cocci during the lag and stationary phase of growth (Luscombe and Gray, 1971; Forni and Grilli Caiola, 1992).

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The role played by *Arthrobacter* in the association is unknown, although it has been suggested that the bacteria can produce and excrete the plant hormone indole-3-acetic acid in the leaf cavities (Forni et al., 1992a) and they may also contribute to the mucilage production of the algal packets (Forni et al., 1992b). Moreover, an involvement in the N_2-fixation activity has been hypothesized by Lindblad et al. (1991) based on immunogold labeling of nitrogenase in the bacteria.

Petro and Gates (1987) suggested the possibility of a controlled situation between *Azolla*, *Anabaena* and *Arthrobacter* based on the ubiquity and the constant bacterial population densities. However, controversy still exists about the bacterial population of *Azolla*, since some authors (Plazinski et al., 1990; Nierzwicki-Bauer and Aulfinger, 1990) recently claimed the presence of additional bacterial genera in the leaf cavities of the fern.

In order to better clarify the genus allocation of the bacteria living in *Azolla*, the use of polyclonal antibodies may be helpful not only for the taxonomic determinations of the bacteria but also for the revelation of the homogeneity of the bacterial strains isolated from the leaf cavities and from the sporocarps of the same species of the fern, as well as from different species of *Azolla*. In this paper we report data from immunological studies with different *Arthrobacter* strains isolated from *Azolla* and the type strain *Arthrobacter globiformis* ATCC 8010. The enzyme-linked immunosorbent assay (ELISA) was used and the antigenic similarity between the rod and coccus forms of *Arthrobacter* was determined. Furthermore, immunogold labeling was performed in order to verify the presence of *Arthrobacter* in the leaf cavities.

2. Materials and Methods

Plants

Azolla filiculoides Lam. plants were grown in Hoagland, nitrogen-free medium (Van Hove et al., 1983) at "Tor Vergata" University of Rome, under a 6:18 hr (D:L) photoperiod at 25° C and illuminated at 50 μmol $m^{-2}s^{-1}$.

Bacterial strains

The *Arthrobacter* strains used, isolated from the leaf cavities and the sporocarps of *Azolla* (Forni et al., 1989; Forni et al., 1990), are listed in Table 1. The strains belong to the collection of the Department of Biology of the University "Tor Vergata" of Rome.

The type strain *A. globiformis* ATCC 8010 was a gift of the Pasteur Institute of Paris. *Azospirillum brasilense* Tarrand, Krieg and Döbereiner, *Rhizobium leguminosarum* Frank and *Agrobacterium rhizogenes* Riker were kindly

Table 1. Strains of *Arthrobacter* isolated from *Azolla* used as antigens in the immunological tests

Strains antigens	*Arthrobacter* species	Isolated from *Azolla*	
AF1	*A. globiformis* Conn and Dimmick	*A. filiculoides* Lam	(l.c.)*
AF2	*A. globiformis* Conn and Dimmick	*A. filiculoides* Lam	(l.c.)
L2	*A. nicotianae* Giovannozzi-Sermanni	*A. filiculoides* Lam	(l.c.)
A3	*A. globiformis* Conn and Dimmick	*A. filiculoides* Lam	(l.c.)
M1	*A. nicotianae* Giovannozzi-Sermanni	*A. filiculoides* Lam.	(mg)**
A5	*A. globiformis* Conn and Dimmick	*A. mexicana* Presl.	(l.c.)
B1	*A. globiformis* Conn and Dimmick	*A. caroliniana* Willd.	(l.c.)
A4	*A. globiformis* Conn and Dimmick	*A. microphylla* Kaulf.	(l.c.)
E1	*A. crystallopoietes* Ensign and Rittenberg	*A. pinnata* R. Brown.	(l.c.)

* l.c. = leaf cavity
** mg = megasporocarp

supplied by Prof. Del Gallo, Department of Fruit Technology, Agro and Environmental Sciences, University of Molise, Campobasso (Italy). *Escherichia coli* Migula was a gift of Prof. Calef, Department of Biology, University of Rome "Tor Vergata", while *Bacillus subtilis* Conn was provided by Dr. Zagaglia, Microbiology Institute, University "La Sapienza" of Rome.

Media and bacterial growth

Arthrobacter strains were maintained on TRN medium (Forni et al., 1989). Cultures of rod or coccus forms were obtained using the procedure of Ensign (1970). *Azospirillum brasilense* and *B. subtilis* were grown on Nutrient broth (Difco), while *Rhizobium leguminosarum* and *Agrobacterium rhizogenes* were grown on YMB and YEM, respectively (Jordan, 1984). *E. coli* was maintained on LB (Maniatis et al., 1982). Protein concentration was determined according to Lowry et al. (1951). Bovin serum albumin was used as standard.

Preparation of antisera

Arthrobacter strains AF1, AF2, and the type strain ATCC 8010 were grown in liquid culture. AF2 was grown as rod (AF2r) and as coccus forms (AF2c). The other strains were rods. The bacterial cells were heated for 30 min at 60° C, centrifuged, washed in phosphate-buffered saline (PBS; 20 mM phosphate buffer, pH 7.4, supplemented with 150 mM NaCl) and resuspended in PBS to a final density of 10^8 bacteria/ml.

Four rabbits were injected intramuscular with 2 ml of a 1:1 mixture of bacteria and incomplete Freund adjuvant. Two booster injections of bacteria suspended in PBS were administered every 2 weeks thereafter. The final bleed

for antisera was carried out 7 days after the last injections. The blood was allowed to clot and the sera, obtained by centrifugation at 6000 g for 20 min at 4° C, in dilution 1:100, were used for the immunological tests.

Enzyme Linked Immuno-Sorbent Assay (ELISA)

PVC plates (3911 Falcon Labware, Division of Becton Dickinson & Co., Oxnard, Ca., USA) were coated using bacterial cells (8 μg/well). The cells were then incubated with 35% H_2O_2 in PBS (1:500) in order to reduce any endogenous peroxidase activity. After 30 min at room temperature, the plates were washed three times by centrifugation with PBS and incubated with a solution of PBS containing 0.25% gelatin, 3% skimmed dried milk and non-specific goat polyclonal immunoglobulins (1:50). After 45 min at room temperature, the plates were again washed three times as above. Rabbit anti-*Arthrobacter* polyclonal antisera (100 μl) in different dilutions were added to the antigen-coated plates. Negative controls were obtained by incubating the same antigen with a PBS and preimmune serum. After 45 min, the plates were washed and the binding of the antibodies was detected by color development on addition of 25 μl horse radish peroxidase-conjugated goat anti-rabbit immunoglobulins (Bio-Rad Laboratories, 1000 fold diluted) as secondary antibody. Thirty min later the wells were rinsed three times with PBS, and 100 μl of o-phenylendiamine substrate (0.25 mg/ml in citrate/phosphate buffer, 0.4 μg/ml 30% H_2O_2) was added to detect residual peroxidase activity dependent on the quantity of bound antibodies. The colorimetric reaction was blocked by adding 100 μl/well 2 M HCl. Optical densities at 492 nm were determined from each well with an automatic ELISA reader (EASY E.I.A., GIO DE VITA S.p.A. Rome, Italy).

Immunogold labeling and transmission electron microscopy (TEM)

Bacterial cells and *Azolla filiculoides* plants were fixed in 2.5% glutaraldehyde in 0.2 M Na-Cacodylate buffer, pH 7.2, overnight at 4° C, dehydrated in a graded ethanol series and propylene oxide, and embedded in Epon 812 resin. Thin sections were mounted on 200-mesh nickel grids. Immunogold labeling was performed as reported in Canini et al. (1992). The antisera was diluted 1:100 and the secondary antibody was goat anti-rabbit IgG conjugated to 10 nm colloidal gold particles (Bio Cell, Research Laboratories, Cardiff, UK) diluted 1:20. Controls were performed by omitting the primary antibody and using preimmune serum instead of antiserum. The grids were observed with a Zeiss CEM 902 electron microscope at 80 KV.

3. Results

Immunological characterization

Polyclonal antisera, produced against the bacterial isolates AF1 and AF2 in the rod and coccus forms and against the type strain *A. globiformis* ATCC 8010 were used in order to detect the homologies among the antigens of the bacteria isolated from the fern and classified as *Arthrobacter* and the type strain of the same genus (Table 2). The antibodies against *A. globiformis* ATCC 8010 cross-reacted with the two bacterial strains isolated from *Azolla filiculoides*.

In a subsequent test the same antibodies were tested against gram-positive and gram-negative bacteria belonging to different genera and species (Table 3). No antigenic reaction was found between the anti-AF1, anti-AF2 and anti-ATCC 8010 and the non-*Arthrobacter* bacterial genera.

The cross-reactivity between the antiserum against ATCC-8010 and several *Arthrobacter* strains (Table 1) isolated from the leaf cavities and from the megasporocarps of different species of *Azolla* was tested. The data, presented

Table 2. Immunological cross-reaction between the type strain *Arthrobacter globiformis* ATCC 8010 and the bacterial strains isolated from *Azolla filiculoides*

Antigen	Absorbance					
	Antiserum					
	anti-ATCC	anti-A1	anti-Af2r*	anti-Af2c*	PBS	Pr. ser*.
ATCC	0.051	0.0303	0.0344	0.0324	0.000	0.001
AF1	0.0434	0.0455	0.0393	0.0380	0.000	0.000
Af2r	0.0543	0.0491	0.0547	0.0485	0.000	0.000
AF2c	0.0503	0.0491	0.0487	0.0510	0.000	0.000

* r = rods; c = cocci; pr. ser. = preimmune serum

Table 3. Immunological cross-reactions between anti-ATCC, anti-AF1 and anti-Af2r and gram-positive and gram-negative bacteria

Bacterial antigen	Absorbance				
	anti-ATCC	anti-Af1	anti-AF2r	PBS	Pr. ser*.
gram-positive:					
A. globiformis ATCC	0.0555	0.0412	0.0393	0.000	0.0012
B. subtilis	0.0028	0.0030	0.0019	0.000	0.000
gram-negative:					
Agrobacterium	0.0027	0.0024	0.0013	0.000	0.000
Azospirillum	0.0041	0.0040	0.0015	0.000	0.000
E. coli	0.0001	0.0012	0.0003	0.000	0.000
Rhizobium	0.0000	0.0000	0.0000	0.0000	0.0000

* pr. ser. = preimmune serum

Table 4. Immunological cross-reactions between *Arthrobacter* species isolated from different *Azolla* species

Arthrobacter species	Isolated from *Azolla*	Absorbance		
		anti-ATCC	PBS	Pr. ser.
A. globiformis (ATCC)	–	0.0810	0.000	0.0021
A. globiformis (AF1)	*A. filiculoides*	0.0524	0.000	0.0011
A. globiformis (AF2)	*A. filiculoides*	0.0568	0.000	0.0014
A. globiformis (A3)	*A. filiculoides*	0.0598	0.000	0.0004
A. globiformis (A4)	*A. microphylla*	0.0553	0.000	0.0000
A. globiformis (A5)	*A. mexicana*	0.0577	0.000	0.0000
A. globiformis (B1)	*A. caroliniana*	0.0567	0.000	0.0000
A. nicotianae (M1)	*A. filiculoides*	0.0386	0.000	0.0029
A. nicotianae (L2)	*A. filiculoides*	0.0375	0.000	0.0000
A. crystallopoietes (E1)	*A. pinnata*	0.0354	0.000	0.0000

in Table 4, indicated a cross-reactivity between antibodies against ATCC and the bacteria isolated from the different species of *Azolla*. A weaker cross-reaction was detected to *Arthrobacter* species, different from the type species *A. globiformis*, i.e. *A. nicotianae* and *A. crystallopoietes*.

Immunogold labeling

Immunogold labeling was performed in order to visualize the antigenic reactions between the antibodies against the type strain ATCC 8010 and the bacteria isolated from *Azolla*. A cross-reaction was observed between the antibodies ATCC 8010, and strain B1 (*A. globiformis*) isolated from the leaf cavities of *A. caroliniana* (Figs. 1, 2). Although the type strain reacted preferentially with its homologous antiserum, there was a specific cross-reaction with the other *Arthrobacter* strains both in ELISA and in immunogold labeling. Moreover, the antiserum obtained against the coccus reacted with the rod, confirming the antigenic similarity of the two morphological forms of *Arthrobacter* (Fig. 3). No significant labeling was detected using preimmune serum (Fig. 4).

The labeling with anti-ATCC of the bacteria in the leaf cavity of *A. filiculoides* confirmed the presence of *Arthrobacter* in the symbiosis even if a different localization of the labeling was observed (Figs. 5, 6). In fact, the labeling with anti-ATCC is localized principally in the cytoplasm (Figs. 5, 6). All the bacteria in the leaf cavities appeared labeled.

4. Discussion

The constant presence of heterotrophic eubacteria in the *Azolla-Anabaena*

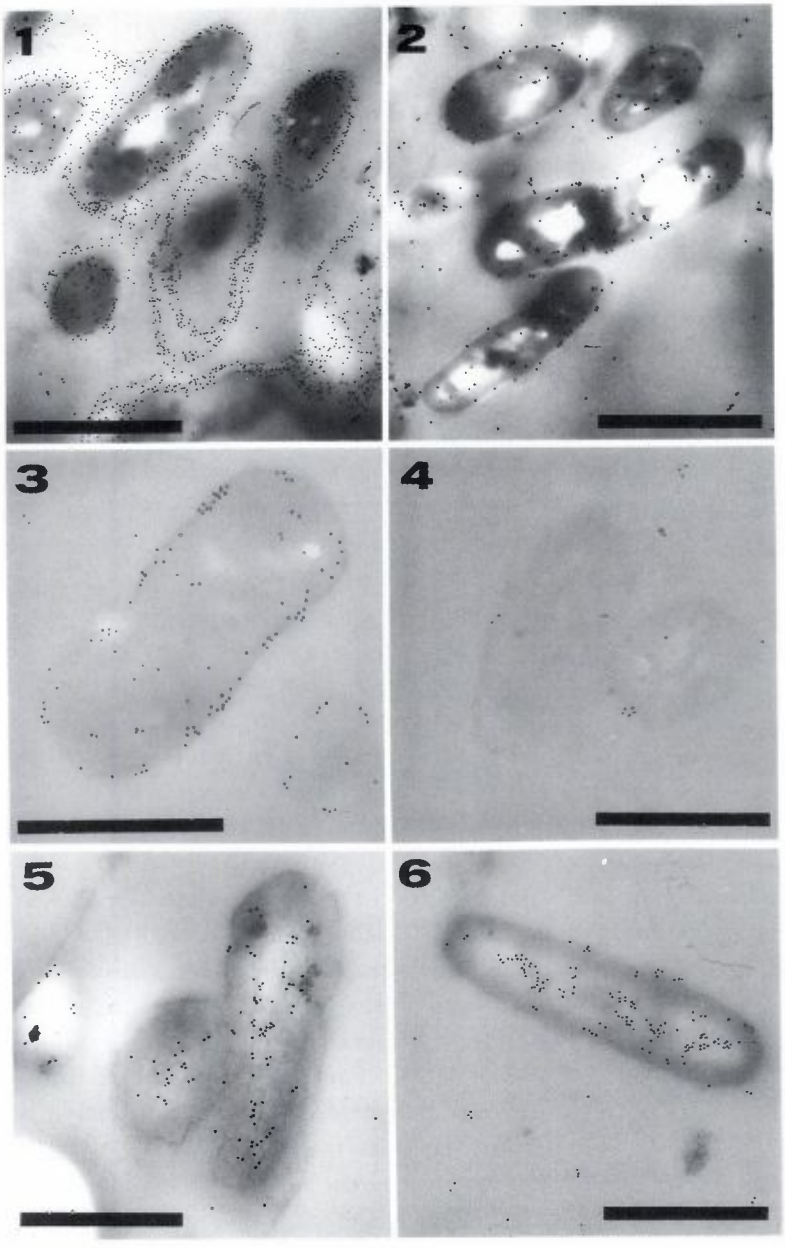

Figure 1. Localization of anti-ATCC in type strain *Arthrobacter globiformis* ATCC 8010. Bar = 1 μm.

Figure 2. Localization of anti-ATCC in *Arthrobacter globiformis* strain B1. Bar = 1 μm.

Figure 3. Anti-ATCC antibody, obtained against coccus forms, localized in the *Arthrobacter* rod. Bar = 0.5 μm.

Figure 4. Immunogold labeling of control preimmune serum in bacteria located within *Azolla* leaf cavities. Bar = 0.5 μm.

Figure 5, 6. Localization of anti-ATCC in bacteria present in *Azolla filiculoides* leaf cavities. Bar = 0.5 μm.

symbiosis suggests the hypothesis of a tripartite association (Petro and Gates, 1987; Forni et al., 1990). Although the taxonomic classification of the bacteria has varied (Plazinski et al., 1990; Nierzwicki-Bauer and Aulfinger, 1990), a high number of *Arthrobacter* has been reported both in leaf cavities (Wallace and Gates, 1986; Petro and Gates, 1987; Forni et al., 1989) and sporocarps (Forni et al., 1990) of different species of *Azolla*.

It has been demonstrated that the indirect ELISA test is able to distinguish bacteria genera and species with a good degree of specificity (Barraquio et al., 1986). Consequently the ELISA method was used in this work as a tool for further identification of the bacterial strains isolated from *Azolla*.

Our immunological studies confirm the previous assignment of the bacteria to the genus *Arthrobacter* (Forni et al., 1989; Forni et al., 1990), although differences in the immunological reactions among the various *Arthrobacter* species were detected. These differences indicate the possibility that *Arthrobacter* can be discriminated at species level using the ELISA test.

Although *Arthrobacter* is considered to be a gram-positive eubacteria, the cultures frequently possess gram-negative cells interspersed with gram-positive ones. Gram-variability has been found even under optimal growth conditions (Beveridge, 1990). The Gram staining reactions of *Arthrobacter* depend on the growth phase, i.e. different percentages of cells react as gram-negative, depending on the growth stage (Beveridge, 1990). According to Ward and Claus (1973) this Gram-staining variability may be due to differences in the cell wall of the bacteria.

Gram variability has also been detected in bacteria isolated from *Azolla* and classified as *Arthrobacter* (Forni et al., 1989; Forni and Grilli Caiola, 1992). However, judging from the electron micrographs, these bacteria should be considered as gram-positive since the cell wall shows a homogenous structure of varying thickness, lacking the trilaminar portion typical of the gram-negative bacteria (Forni and Grilli Caiola, 1992). Therefore, the lack of immunological cross-reaction between *Arthrobacter* strains and gram-negative bacteria represent a further confirmation of the ultrastructural data.

Another characteristic of *Arthrobacter* genus is its pleomorphism, i.e. a rod-coccus life cycle. According to Krulwich et al. (1967a,b) the cell walls of cocci and rods in *A. crystallopoietes* consisted of similar glycopeptides. A common glycopeptide composition in the rod and coccus forms may lead to immunological cross-reaction between the two forms. Both ELISA and immunogold labeling of rods and cocci indicate the presence of common antigens. The isolated bacterial strains and the bacteria present in the leaf cavities of *Azolla filiculoides* were compared using the immunogold method, in order to verify the data obtained with ELISA. The presence of *Arthrobacter* in the

Azolla-Anabaena association was confirmed by the immunogold labeling of the bacteria in the leaf cavities of *Azolla filiculoides* even though a different localization of the labeling was observed between the free-living and symbiotic bacteria. This may be due to a different distribution of the antigenic determinant at different metabolic stages or in different growth conditions of the bacteria. Moreover, the variations in the labeling may be a consequence of differential accessibility of antigenic sites. However, the absence of binding of anti-ATCC to non related bacteria indicated a specificity of the antiserum with *Arthrobacter*.

Although it cannot be excluded that there are different bacteria in *Azolla* species grown in different areas of the world, the data reported in this paper along with the previous ones (Grilli Caiola et al., 1988; Forni et al., 1989; Forni et al., 1990) indicate that the bacteria in the leaf cavities are gram-positive and belong to the genus *Arthrobacter*. Bacteria with morphological characteristics similar to those present in the leaf cavities of *A. mexicana* and *A. caroliniana* (Nierzwicki-Bauer and Aulfinger, 1990; Nierzwicki-Bauer and Aulfinger, 1991) were not observed in our previous studies (Grilli Caiola et al., 1993; Albertano et al., 1993).

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