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The Presence of Lectins in Bacteria Associated with the *Azolla-Anabaena* Symbiosis

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Abstract

Cell extracts from the *Azolla-Anabaena* system are known to cause agglutination of erythrocytes, and if lectins, which are carbohydrate-binding proteins, are involved in the establishment of the *Azolla-Anabaena*-bacteria symbiosis, they may play any role in it. In order to clarify this situation, we have isolated and identified six different bacterial strains contained in the leaf cavities of two *Azolla* species (*A. filiculoides* and *A. pinnata*). From these symbiotic bacteria lectin extracts were prepared, treated with polyvinylpolypyrrolidone (PVPP), and analyzed by SDS-PAGE that, for each analyzed bacterial strain, revealed several protein bands whose molecular masses varied between 50 and 142 kDa. The protein extracts, with and without PVPP treatment, were also used in haemagglutination tests, and showed a positive reaction. The results of this study indicate that lectins are involved in the haemagglutination activity of the extracts recovered from the symbiotic bacteria present in the system *Azolla-Anabaena*.

Keywords: *Azolla*, bacteria, lectins, symbiosis

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1. Introduction

The aquatic pteridophyte of the genus *Azolla* contains a cavity in the dorsal lobe of each of its leaves which has a symbiotic nitrogen fixing cyanobacterium *Anabaena azollae*, and a variety of bacteria (see Braun-Howland and Nierzwicki-Bauer, 1990 for a review). The latter have been referred to as bactobionts (Lindblad et al., 1991).

The bacteria isolated from the *Azolla* leaf cavity have been identified as *Pseudomonas* (Bottomley, 1920), *Alcaligenes faecalis, Caulobacter fusiformis* (Newton and Herman, 1979), coryneform bacteria (Gates et al., 1980) and as members of the genus *Arthrobacter* Conn and Dimmick, on the basis of biochemical tests and morphological data (Forni et al., 1989; Grilli Caiola et al., 1988; Petro and Gates, 1987; Wallace and Gates, 1986). The genus *Arthrobacter* has also been suggested by transmission electron microscopy studies since the detected bacteria showed a rod-coccus morphology as well as the "V" formations characteristic of *Arthrobacter* species (Carrapiço, 1991; Carrapiço and Tavares, 1989). Other studies reported the occurrence of several ultrastructurally distinct types of *Azolla*-associated bacteria where only one of them resembles *Arthrobacter* species (Nierzwicki-Bauer and Aulfinger, 1991), and another was identified as belonging to the genus *Agrobacterium* on the basis of bacteriologic tests and DNA/DNA hybridization analysis (Plazinski et al., 1990). Nevertheless the taxonomic classification of these bacteria remains unclear.

Based on conventional morphological and biochemical features, bactobionts of the genus *Arthrobacter*, have also been observed in the sporocarps of *Azolla*, (Carrapiço, 1991; Forni et al., 1990). This suggests that they were retained throughout the sexual cycle of the fern, with a persistence similar to that of the *Anabaena azollae*, and that they may be considered to be a third partner of this symbiotic association (Carrapiço, 1991; Carrapiço and Tavares, 1989; Petro and Gates, 1987).

Thus, the phenomenon of biological recognition at the symbiont-host interface is essential to the development and understanding of this symbiosis, where the lectins (carbohydrate-binding proteins which agglutinate animal and plant cells) may play any role in the association. Their binding capacities may lead to a variety of biological effects such as adhesion of the bactobionts to the epidermal cells of the *Azolla* leaf cavities and/or the agglutination of the bactobionts. It is known that, by means of erythrocyte agglutination assays and by inhibition of haemagglutination activity with selected carbohydrates, lectins activities were detected in the *Azolla-Anabaena* system, (Ladha and Watanabe, 1984; McCowen et al., 1987; Serrano et al., 1994). Erythrocytes can also be agglutinated by polyphenols (Leizerovich et al., 1988; Razafimahery et al., 1994).

In order to determine if haemagglutination is caused by lectins or by polyphenols, we have repeatedly isolated and purified different bacterial strains involved in the *Azolla-Anabaena* symbiosis, which were subsequently identified, and the protein extracts obtained were treated with an inhibitor of polyphenol-oxidases and subjected to haemagglutination assays.

2. Materials and Methods

Plant material

We have collected *Azolla filiculoides* Lam. from Tejo river channels (Samora Correia region) in Portugal and *Azolla pinnata* R. Br. from the banks of Geba river (Contuboel region) in Guinea-Bissau. This material was immediately packed in plastic bags and kept in a cold-storage container until its utilization in this study. Both species were formally identified in the laboratories of Centre for Environmental Biology, Department of Plant Biology, Faculty of Sciences, University of Lisbon, Portugal.

Isolation and growth of bacteria

The fern leaves were surface sterilized for a period of 10–15 min with 30% sodium hypochlorite, then washed three times with sterilized water, following a modification of the method described by Gates et al. (1980). Single colony isolates were obtained using routine bacteriological procedures, and maintained on TRN medium containing ($g.l^{-1}$): tryptone (Difco) 10; NaCl, 5; agar (Difco) 15 (Forni et al., 1989). The Petri dishes were incubated at 37°C.

To check the adequacy of the procedure for removing surface contaminants, the sterilized leaves were routinely plated on TRN medium and incubated at the same temperature. The absence of bacterial growth after five days indicated a successful surface sterilization (Forni et al., 1990).

Identification of the bacteria

Methods used to determine Gram classification, included Gram staining and the KOH test (Gregersen, 1978). Bacterial identification was based on API (API system S.A., France) and BIOLOG (Biolog Inc., E.U.A.) automated systems.

Preparation of the extracts

For all of the isolated bacterial strains, the cells obtained from previous cultures were incubated for 24 h at 37°C in 5 ml of a TRN medium, and

centrifuged for 10 min at 12,000 xg. The cellular pellets were treated for 30 min at room temperature with 100 µl of an extractive solution for lectins (1 M NaCl, 0.02 M $MnCl_2.4H_2O$ and 0.02 M $CaCl_2.2H_2O$, pH 6.5) (Driessche et al., 1982). After centrifugation as above, the pH of the respective supernatants was adjusted to pH 4.6 with 5 N HCl and brought to 60% $(NH_4)_2SO_4$ saturation by the addition of solid salt. The resulting solutions were stored at 4°C for 12 h, and centrifuged for 20 min at 3,000 xg. The resulting pellets were dialyzed against the above mentioned extractive solution (for lectins) at 4°C, and concentrated to smaller volumes. Finally they were centrifuged for 3 min at 12,000 xg and the respective supernatants were sub-divided in two fractions. One fraction was analyzed by polyacrylamide gel electrophoresis and the other used in haemagglutination tests. In both cases the samples were treated with polyvinylpolypyrrolidone (PVPP) (0.5%, wt/vol).

Polyacrylamide gel electrophoresis

The protein extracts treated with PVPP (0.5%, wt/vol) and without PVPP were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 10% (wt/vol) polyacrylamide resolving gels, according to Laemmli (1970). The protein bands were detected by silver staining (Morrissey, 1981), and molecular masses deduced using standard proteins contained in the Low Molecular Weight kit (Pharmacia): Phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Haemagglutination assay

Each lectin extract was sub-divided into two aliquots. One aliquot was treated with PVPP (0.5%, wt/vol), to bind phenolic compounds (Young et al., 1993), and both were used for the haemagglutination tests. The lectin of *Canavalia ensiformis* (Con A) was used as a standard. This assay was performed by using serial 2-fold dilutions in U-shaped microtiter plates (Nunclon, Denmark). Each well contained 20 µl of diluted test solutions (in the proportion of 1:5 with saline solution), and 20 µl of a 4% suspension of untreated human red blood cells (HRBC). The plates were incubated for 24 h at room temperature (Nicolson, 1974).

3. Results

Six single colony isolates of bacteria were obtained from the leaves of the two *Azolla* species (Table 1). Equivalent results were obtained with three

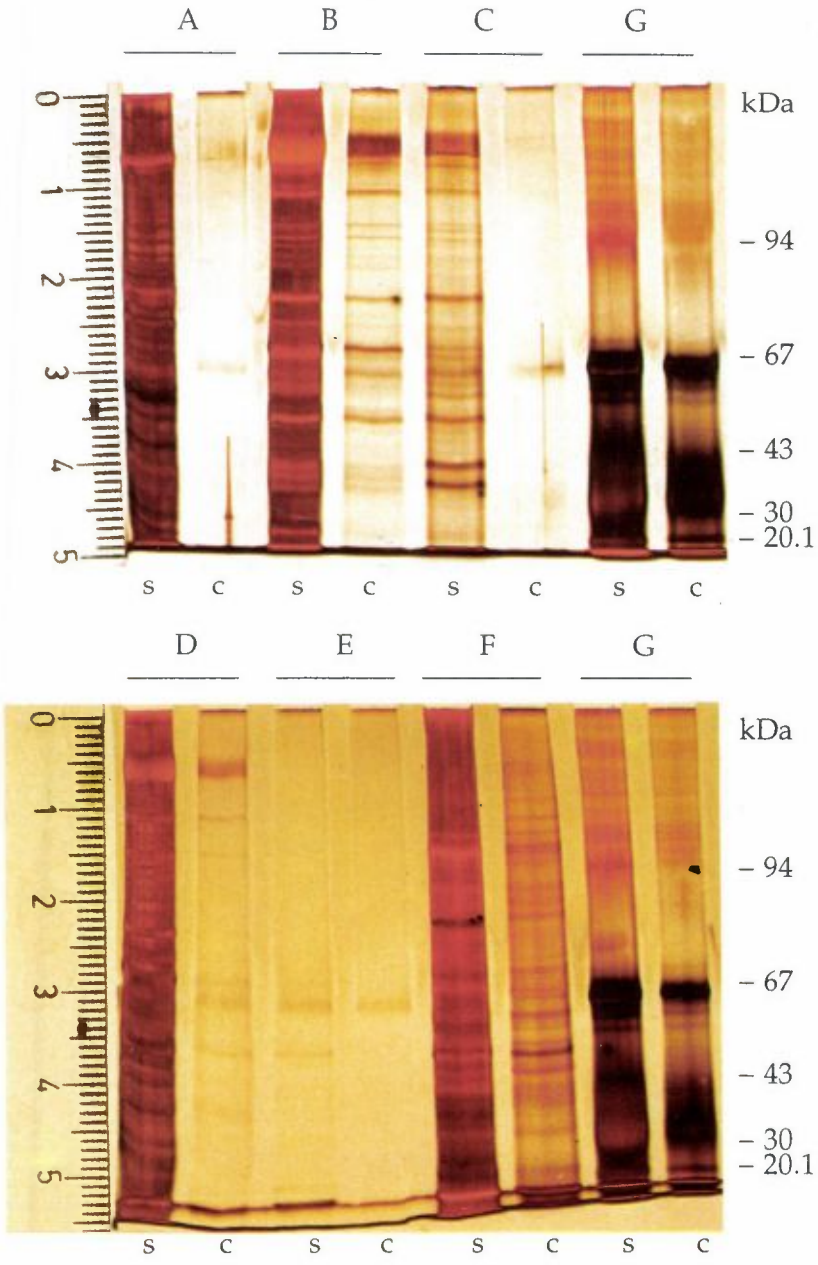

Figure 1. SDS-PAGE of the protein extracts recovered from the studied strains, after PVPP (0.5%, wt/vol) (c) and without PVPP (s) treatment. Lane A, *Agrobacterium radiobacter*; lane B, *Staphylococcus* sp.; lane C, *Rhodococcus* sp.; lane D, *Rhodococcus equi*; lane E, *Corynebacterium jeikeium*; lane F, *Weeksella zoohelcum*; lane G, standard proteins.

Table 1. Characteristics of the bacteria isolated from leaves of *A. filiculoides* (A, B, C, D and E) and of *A. pinnata* (B and F).

Characteristics	Strains A	B	C	D	E	F
Colony color:						
White	+	+	–	–	–	–
Lemon yellow	–	–	+	–	–	–
Orange	–	–	–	+	+	–
Red	–	–	–	–	–	+
Morphology	rods	coccus	rod/coccus	rod/coccus	rods	rods
Gram staining	–	+	+	+	+/–	–
Oxidase	+	–	–	–	+	+
Catalase	+	+	+	+	–	+
Gelatin hydrolysis	–	–	–	–	–	+
Nitrate reduction	+	+	+	+	+	–
β-galactosidase	+/–	–	+	–	–	–
Acid (aerobically) from:						
Saccharose	+/–	–	+	–	+/–	–
Arabinose	+	–	+	–	–	–
Mannitol	+	+	+	–	–	–
Fructose	+	+	+	+/–	–	–
Glucose	+	+	+	–	–	–
Maltose	+	–	+	–	–	–
Starch	+/–	+/–	+	–	+/–	+/–
Rhamnose	+/–	+/–	+	–	–	–
Galactose	+	+/–	+	–	–	–
Mannose	+	+	+	–	–	–
Sorbitol	+	+/–	+	–	–	–
Glycerol	+	+	+	–	–	–
Urease	–	–	–	–	–	+/–
Indole production	–	–	–	–	–	–
Hydrogen sulfide	–	–	–	–	–	–
Voges-Proskauer	–	+	–	–	–	–
Citrate, Simmons	–	–	–	–	–	–
Pirazynamidase	–	–	+	–	+	–
Pyrrolidonylarilamidase	–	–	–	–	+/–	–
Alkaline phosphatase	–	–	–	–	+	+
β–glucuronidase	–	–	–	–	–	–
α-glucosidase	–	–	–	–	–	+
N-acetil-β-glucosaminidase	–	–	–	–	–	+
Esculin hydrolysis	–	–	+	–	+	+/–
Arginin dihydrolase	–	+/–	–	–	–	–
Acid (anaerobically) from:						
Glucose	n.d.	n.d.	–	–	+	n.d.
Ribose	n.d.	n.d.	–	–	–	n.d.
Xylose	n.d.	n.d.	–	–	–	n.d.
Mannitol	n.d.	n.d.	–	–	–	n.d.
Maltose	n.d.	n.d.	–	–	+	n.d.
Lactose	n.d.	n.d.	–	–	–	n.d.
Saccharose	n.d.	n.d.	–	–	+	n.d.
Glycogen	n.d.	n.d.	–	–	+	n.d.

n.d. = not determined; + = positive reaction; – = negative reaction; +/– = intermediate reaction.

independent tests on isolation. Based on the bacterial profiles obtained, using the API and BIOLOG systems, isolates were identified as *Agrobacterium radiobacter* (A), *Staphylococcus* sp. (B), *Rhodococcus* sp. (C), *Rhodococcus equi* (D), *Corynebacterium jeikeium* (E), and *Weeksella zoohelcum* (F).

Fig. 1 shows the protein profiles of the six strains obtained by SDS-PAGE. For each sample, the gels revealed some protein bands that remained after the PVPP treatment. These bands are bacterial lectins with the molecular masses of 65.2 and 132.1 kDa (for strain A), 53.7, 68.5, 81.2, 117 and 138.7 kDa (for strain B), 68.5 kDa (for strain C), 60.6, 65.2, 117 and 142.1 kDa (for strain D), 65.2 kDa (for strain E), and 49.9, 57.8, 62.1, 81.2 and 101 kDa (for strain F).

In the haemagglutination assays with HRBC, all the protein extracts treated with PVPP (0.5%, wt/vol) and without PVPP treatment showed a positive haemagglutination reaction.

4. Discussion

The number of bacteria isolated from the leaves of *A. pinnata* was fewer than from *A. filiculoides*. Minor numbers of bacteria isolated from *A. pinnata* were also achieved by other researchers (Braun-Howland and Nierzwicki-Bauer, 1990).

Several authors have identified the bacteria isolated from *Azolla* as a species of the genus *Arthrobacter* (Forni et al., 1990; Grilli Caiola et al., 1988; Wallace and Gates, 1986), although the taxonomic classification of these organisms remains controversial (Gates et al., 1980; Nierzwicki-Bauer and Aulfinger, 1991). The Gram staining and the morphology of strains C and D, isolated from the leaf cavities of *A. filiculoides*, are similar to the type strains of the genus *Arthrobacter*, reported by those authors (Forni et al., 1990; Grilli Caiola et al., 1988; Wallace and Gates, 1986), but API and BIOLOG systems revealed the genus *Rhodococcus* as the most related with strains C and D.

The identification by the API and BIOLOG tests of the isolated strain A (as *Agrobacterium radiobacter*) from *A. filiculoides* is of interest because of a similar report on the isolation of *Agrobacterium* sp. from the same *Azolla* species (Plazinski et al., 1990).

The tests for identification of the isolated strains B (present in *A. filiculoides* and *A. pinnata*), E (from *A. filiculoides*) and F (from *A. pinnata*) showed metabolic differences among them. The species *Staphylococcus* sp., *Corynebacterium jeikeium* and *Weeksella zoohelcum* were chosen by the identification systems as the most related strains, respectively. As these three species were never found within the leaf cavity of *A. filiculoides* and *A. pinnata*, the data obtained with API and BIOLOG tests suggest that other genera of bacteria yet unidentified may there exist.

Lectins are involved in a wide range of interactions between prokaryotes and eukaryotes. The main reason for the current intense interest in microbial lectins is the evidence that many of them may play a crucial role in mediating adherence to surfaces colonized by the microorganism (Mirelman and Ofek, 1986). Indeed, microbial surface lectins are now considered to be determinants of virulence in the infection of both animals and plants (Mirelman and Ofek, 1986). On the other hand, microbial lectins have not yet found uses as biochemical probes, as is the case with plant lectins, that are widely used in biochemical research.

If lectins play any role in the recognition process between *Azolla* and the symbionts, the manner in which they do so has yet to be resolved. Conventional haemagglutination techniques have been used to demonstrate the presence of lectins in cyanobacteria freshly isolated from *Azolla* species. Although it has been suggested that lectins are associated with the cyanobacterial partner (Kobiler at al., 1981; Kobiler et al., 1982), the majority of reports have indicated that haemagglutination activity is specifically associated with the fern (Ladha and Watanabe, 1984; McCowen et al., 1987; Mellor et al., 1981).

The inhibition of lectin haemagglutination activity in the presence of selected carbohydrates was obtained with 50 mM to 200 mM of D-fructose, D-galactose, D-glucose, D-lactose and D-saccharose for isolates A, B, C, D and F, from *A. filiculoides* and *A. pinnata*. For strain E, D-saccharose did not inhibit the agglutination. No inhibition of lectin haemagglutination activity was detected with D-fucose, D-manose and L-rhamnose at 100 mM and 200 mM (Serrano et al., 1994).

The addition of PVPP has been used for the removal of phenolic compounds which could contaminate the plant extracts, as the PVPP forms hydrogen bonds with phenolic compounds, originating a PVPP-phenolic precipitate which is removed from the treated extract by centrifugation. This strategy, having been also adapted for DNA purification protocols (Young et al., 1993), was followed in this study.

The electrophoretic profiles of the six strains isolated, as detected by SDS-PAGE, showed a differential protein reactivity with PVPP, with the exception of the lectin bands. A positive haemagglutination reaction was obtained for all the protein extracts of the studied strains, with and without PVPP treatment.

These data indicate that the protein extracts from the bacterial strains isolated from the *Azolla* leaf cavity, contain an agent causing haemagglutination. If lectins are this agent, they may play any role in the recognition process between the partners of the *Azolla-Anabaena* association. The manner in which they do so has yet to be resolved. Further studies on other possible physiological roles of these molecules, such as their participation in the prokaryotes life cycle and *Azolla* development, shall provide as an excellent model for research works.

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