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DNA Base Composition of Eubacteria Isolated from Four Species of *Azolla*

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Abstract

Eubacteria, similar to previously described *Azolla*-associated *Arthrobacter*, were isolated from four species of *Azolla*: *A. caroliniana*, *A. filiculoides*, *A. mexicana*, and *A. miocrophylla*. The DNA base composition (G + C content) of 20 eubacterial isolates, five from each fern species, ranged from 57.7% to 68.2%. Nineteen of the twenty isolates had a G + C content greater than 59% and therefore were within the reported range for œspecies. Although all major phenotypic characteristics of the isolates were similar, statistical analyses indicated that each fern species contained at least two groups based on G + C content (p=0.1). These data are not inconsistent with reports that the predominant *Azolla*-associated eubacteria are arthrobacters. They do suggest, however, that at least different biotypes of *Arthrobacter* and perhaps even other unidentified genera of eubacteria may exist within a leaf cavity of a single *Azolla* species.

Keywords: *Azolla*, *Arthrobacter*, DNA base composition, leaf-cavity bacteria

1. Introduction

The symbiotic relationship between the aquatic fern, *Azolla*, and *Anabaena azollae*, the cyanobacterium which is incorporated into cavities which form in the fern's dorsal leaf lobes has been well characterized (reviewed by Peters and Meeks [1989]). The heterocystous cyanobacterium provides the fern with a

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source of fixed nitrogen, while the fern provides the cyanobiont with a specialized niche and nutrients. In addition to the cyanobiont, eubacteria also are present in *Azolla* leaf cavities (Newton and Herman, 1979; Peters et al., 1978; Gates et al., 1980; Wallace and Gates, 1986; Petro and Gates, 1987; Grilli Caiola et al., 1988; Plazinski et al., 1990; Nierzwicki-Bauer and Aulfinger, 1990).

The eubacteria have been visualized in the leaf cavities via electron microscopy (Nierzwicki-Bauer and Aulfinger, 1990), enumerated following extraction from leaf cavities (Petro and Gates, 1987) and characterized with biochemical and physiological tests (Wallace and Gates, 1986; Grilli Caiola et al., 1988; Gibson et al., 1991). Eubacteria isolated from four species of *Azolla* were identified as members of the genus *Arthrobacter* Conn and Dimmick by Wallace and Gates (1986) on the basis of biochemical tests of the bacterial wall. Grilli Caiola et al. (1988) subsequently identified eubacteria from *A. caroliniana* as *Arthrobacter* using morphological and biochemical data. These isolates, which stain gram-positive for a brief period during log phase but appear gram-negative thereafter, exhibit the rod-coccus growth cycle and "V" formations characteristic of *Arthrobacter*. Nevertheless, the taxonomic placement of these organisms remains unclear. Recent reports have challenged the classification of these bacteria as *Arthrobacter* spp. on the basis of morphology, ultrastructure, and the mode of cell division, and suggested the occurrence of different general within the *Azolla* leaf cavities. Nierzwicki-Bauer and Aulfinger (1990, 1991) reported the occurrence of several ultrastructurally distinct types of eubacteria in *A. caroliniana* and *A. mexicana*, but concluded that only one of them resembles *Arthrobacter* species. In another recent study, Plazinski et al. (1990) characterized eubacteria from *A. filiculoides* as members of the genus *Agrobacterium* on the basis of bacteriologic multitest results and DNA/DNA hybridization analysis.

The purpose of this study was to elucidate further the classification of *Azolla*-associated eubacteria. The genomic DNA base composition of isolates from four species of *Azolla* were determined and the mole percentage guanine + cytosine (mol% G + C) of the isolates were compared to one another and to those of other selected bacteria. The mol% G + C is a constant number which is characteristic of pure DNA extracted from an organism. Therefore, comparison of mol% G + C is a standard method for determining the degree of genetic relatedness between two organisms. While organisms with large differences in mol% G + C values may be considered different, organisms with the same or similar values are not necessarily closely related. Nevertheless, organisms which share a number of phenotypic similarities and similar mol%

G + C ratios may be considered to be members of the same genus or species (Johnson, 1984).

2. Materials and Methods

Stock cultures of *Azolla* were obtained from Dr. Gerald Peters, Department of Biology, Virginia Commonwealth University. *Azolla filiculoides* Lam., *A. caroliniana* Willd., *A. miocrophylla* Kaulfaus, and *A. mexicana* Presl. were grown in greenhouse buckets containing International Rice Research Institute (IRRI) medium (Peters et al., 1980). Stock cultures of *Arthrobacter* species obtained from the American Type Culture Collection (ATCC) in Rockville, Maryland included: *Arthrobacter globiformis* ATCC 8010, *A. atrocyaneus* ATCC 13752, and *A. pascens* ATCC 13346. These organisms and *Agrobacterium tumefaciens* strain C58 (provided by Dr. Joseph Formica, Department of Microbiology and Immunology, Medical College of Virginia of Virginia Commonwealth University) were maintained on nutrient agar slants and transferred to fresh media every two weeks.

Isolation of eubacteria

Eubacteria were aseptically isolated from the leaf cavities of *Azolla filiculoides*, *A. caroliniana*, *A. mexicana*, and *A. miocrophylla* using the technique of Petro and Gates (1987). Surface sterilized leaves were homogenized and serial dilutions were plated on Difco Plate Count Agar (PCA) (Difco Laboratories, Detroit, MI). After incubation at 30° C for 3–5 days, five single colony isolates from each fern species were selected and maintained on PCA slants.

Dry extraction

Fifty ml starter cultures of Plate Count Broth (PCB) containing 5.0 g/l tryptone, 2.5 g/l yeast extract, and 1.9 g/l dextrose were inoculated with each of the isolates from the four fern species and incubated until turbid in a New Brunswick gyratory environmental shaker (150 rpm) at 30° C. Fernbach culture flasks containing 1 l PCB were inoculated with 50 ml starter culture and incubated as described above. Late log phase cells were harvested by centrifugation for 10 min at 8,000×g in a Beckman model J-21C centrifuge. Cell pellets were washed twice with 50 mM potassium phosphate buffer (KPi), pH 7.2. Cell yield was approximately 2–3 grams wet weight per liter of culture. Cell pellets were used immediately or frozen at −4° C for later use. No more than one week elapsed between the freeze data and the extraction date.

The method of Johnson (1985), incorporating modifications recommended by the author (John L. Johnson, Virginia Polytechnic Institute and State University, Blacksburg, VA, personal communication), was used for DNA extraction. Washed cells were suspended in an osmotic buffering solution containing 0.35 M sucrose. Cell walls were digested by addition of dry lysozyme and cells were lysed in buffer containing proteinase K (Sigma Chemical Co.). This solution was subjected to phenol/chloroform extractions, which were continued until the aqueous layer was clear and little if any protein interface existed. Then the aqueous layer was removed with an inverted 5 ml pipette and DNA was selectively precipitated with the addition of two volumes of room temperature isopropanol.

RNA was degraded using an RNase mixture containing 1 mg/ml RNase A and 200 units/ml RNase T_1. Following extraction with phonol-chloroform, DNA strands were precipitated with 95% ethanol and harvested on a glass rod. The dried DNA was suspended in 0.1×standard saline citrate (SSC).

Determination of mole% gluanine + cytosine

The mol% G + C of each isolate was determined using a Gilford Model 2400 spectrophotometer (Gilford Instrument Co., Oberlin, Ohio) equipped with an electronically heated cuvette holder, and thermoprogrammer. Thermal melting (Tm) determinations were carried out in 0.1×SSC to Tm values in SSC. All Tm determinations were repeated at least twice for each DNA sample. Data were subjected to statistical analysis using a one way ANOVA and a Sheffe's test (Zar, 1984).

Reagents

All inorganic salts and reagents used in DNA extraction procedures were of molecular biology-grade and purchased from either Sigma Chemical Co., or Fisher Chemical Co., Fairlawn, NJ. Tryptone, yeast extract, and nutrient agar were purchased from Difco Laboratories, Detroit, MI.

3. Results

Extraction of eubacteria from Azolla

Eubacteria were extruded from the leaf cavities of four species of *Azolla*: *A. caroliniana*, *A. filiculoides*, *A. mexicana*, and *A. miocrophylla*. Serial dilution plates of the material extruded from each species of *Azolla* were incubated at 30° C. Bacterial colonies were visible within 3–4 days. Five single colony isolates were selected at random from dilution plates of each fern.

Based on serial dilutions, the numbers of eubacteria present in the apex and first six leaves of each fern species varied. Of the four species used in this study, *A. mexicana* yielded the lowest number of bacteria, about 2800 organisms/frond segment. The approximate number of bacteria recovered from the other fern species were as follows: 8000/frond segment from *A. miocrophylla*; 140,000/frond segment from *A. caroliniana*; and, 210,000/frond segment from *A. filiculoides*.

Virtually all of the bacterial colonies on the PCA plates, regardless of the fern species, were round, smooth, and moist. Young colonies were off-white and translucent but progressively darkened to an amber color and became increasingly more opaque with age. The only exception was one isolate from *A. miocrophylla* (AM-5) which produced a slimy white colony. This isolate also produced a brown, diffusible pigment when cells were grown on PCA slants. AM-5 was present in the leaf cavities at a much lower frequency than the dominate colony type, accounting for about 10% of the organisms recovered by serial dilution plating of *A. miocrophylla*. Although it initially was difficult to distinguish AM-5 from other isolates on serial dilution plates, older colonies of AM-5 on PCA slants were distinctly different.

Gram stains of all isolates showed predominantly safranin stained cells although some cells on each smear exhibited crystal violet stained granules in otherwise gram-negative appearing cells. All isolates exhibited the pleomorphic rod forms characteristic of *Arthrobacter* and other coryneform bacteria. Rod morphology predominated in cultures during all stages of growth but coccoid forms were present in older cultures. Freshly isolated eubacteria were shown to be non-fermentative when cultured on lactose, sucrose, and dextrose. Isolates were routinely tested on triple sugar-iron agar slants to confirm their inability to ferment these carbohydrates.

DNA base composition

DNA was extracted from the twenty eubacterial isolates: AC-1 through 5 from *A. caroliniana*, AF-1 through 5 from *A. filiculoides*, AX-1 through 5 from *A. mexicana*, and AM-1 through 5 from *A. miocrophylla*. Cell pellets (ca. 2 g wet weight) of cultures grown in one liter PCB and harvested in late log phase provided an ample cell suspension for DNA extraction procedures.

High molecular weight genomic DNA was extracted from all organisms as described in Materials and Methods. The thermal melting midpoint (Tm) and the calculated mol% G + C of the DNA from *Arthrobacter globiformis* ATCC 8010, *A. atrocyaneus* ATCC 13752, and *A. pascens* ATCC 13346 were

Table 1. Tm and base composition of DNA from *Arthrobacter globiformis*, *Arthrobacter pascens*, *Arthrobacter atrocyaneus*, and *Agrobacterium tumefaciens* determined in 0.1×SSC

Bacteria	TM (°C)	Mol% G + C	Reported range
Arthrobacter globiformis ATCC 8010	82°	64.8%	62–65.5%[a]
Arthrobacter pascens ATCC 13346	81.1°	63.1°	63.7%[a]
Arthrobacter atrocyaneus ATCC 13752	85.5°	69.5%	69.5–70.3%[a]
Agrobacterium tumefaciens Strain C58	79.2°	57.9%	57.0–63.0%[b]

[a] Gherna, R.L. Department of Bacteriology, ATCC. Rockville, MD. (personal communication)
[b] Kersters and De Ley, 1984

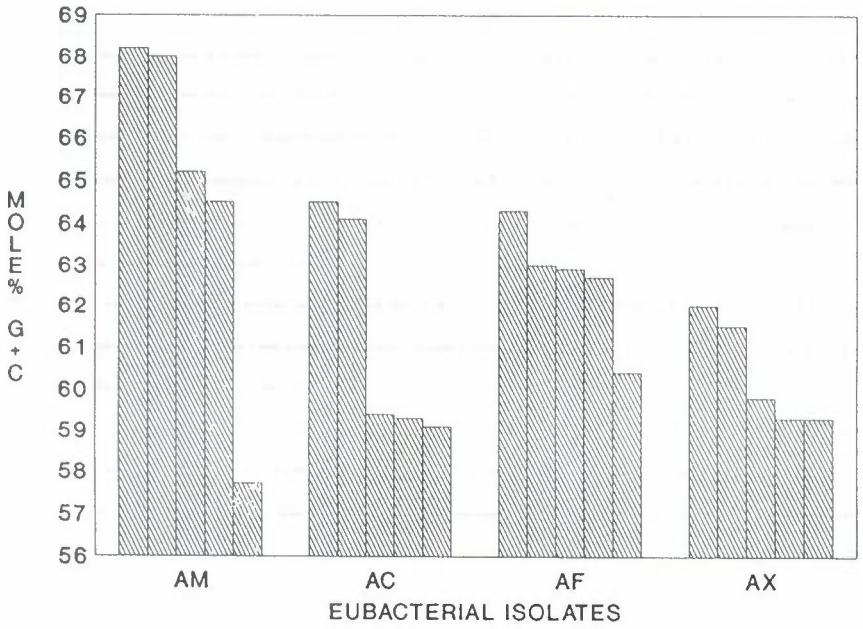

Figure 1. Distribution of DNA base composition of five eubacterial isolates from each fern species: *Azolla microphylla* (AM), *Azolla caroliniana* (AC), *Azolla filiculoides* (AF), and *Azolla mexicana* (AX)

Table 2. Tm and base composition of DNA from eubacterial isolates of *Azolla caroliniana* (AC), *Azolla mexicana* (AX), *Azolla filiculoides* (AF), and *Azolla microphylla* (AM)

Eubacterial isolate	Mean Tm	G + C Content (mol%) and standard deviation
AF-1	80.0°	64.3±0.2
AF-2	79.6°	62.9±0
AF-3	79.5°	63.0±0.5
AF-4	79.4°	62.7±0.4
AF-5	80.6°	60.4±0.2
AX-1	79.8°	59.3±0
AX-2	80.3°	59.8±0.4
AX-3	80.0°	59.3±0.4
AX-4	80.7°	61.5±0.3
AX-5	81.0°	62.0±0
AC-1	80.0°	59.4±0.6
AC-2	80.0°	59.3±0.3
AC-3	79.9°	59.1±0.4
AC-4	82.1°	64.1±0.3
AC-5	82.2°	64.5±0.1
AM-1	83.7°	68.2±0.2
AM-2	82.0°	64.5±0.1
AM-3	82.3°	65.2±0.5
AM-4	83.7°	68.0±0
AM-5	79.2°	57.7±0.1

in close agreement with the values provided by Dr. Robert Gherna, Department of Bacteriology at the American Type Culture Collection, Bethesda, MD (Table 1). The mol% G + C of DNA from *Agrobacterium tumefaciens* strain C58 (57.7%) was within the range reported in *Bergey's Manual of Systematic Bacteriology* for this species (Kersters and De Ley, 1984).

The Tm values for DNA, in 0.1×SSC, extracted from eubacterial isolates of *Azolla* ranged from a low temperature of 79.2° to a high of 83.7°. The mean Tm of multiple determinations for each isolated are listed in Table 2. The mol% G + C was calculated from each individual TM determination and the mol% G + C values for each organism (from two, three or more thermal melting determinations) were averaged and standard deviations calculated. All mol% G + C values were compared using a standard one way ANOVA and the Scheffe's Test.

The mean mol% G + C value and the standard deviation for each of the 20 isolates are reported in Table 2. The mol% G + C values for the 20 isolates ranged from 57.7% to 68.2% (Table 2 and Fig. 1). Statistical analysis of these data using the Scheffe's Test reveals that variation does exist among the

isolates and that, based on mol% G + C, each fern species contains at least two distinct groups of eubacteria. The grouping of isolates from each fern species according to their DNA base composition is apparent in Fig. 1.

4. Discussion

The occurrence of eubacteria in *Azolla* leaf cavities has been reported widely (Gates et al., 1980; Peters et al., 1978; Grilli Caiola et al., 1988; Nierzwicki-Bauer and Aulfinger, 1990) and, although several authors have identified the organisms isolated from *Azolla* as arthrobacters (Wallace and Gates, 1986; Grilli Caiola et al., 1988), the classification of these organisms remains controversial. The morphological and biochemical characteristics of *Azolla*-associated eubacteria isolated in this study from *A. miocrophylla*, *A. caroliniana*, *A. filiculoides*, and *A. mexicana* are similar to those eubacteria obtained from *A. caroliniana*, *A. filiculoides*, *A. mexicana* and *A. pascens* by Wallace and Gates (1986) and Petro and Gates (1987). These bacteria are non-fermentative, pleomorphic rods, which appear predominantly gram-negative on Gram stained smears and produce white/tan colonies on plate count agar.

The estimated numbers of these bacteria associated with a particular fern species and the growth characteristics of our isolates are similar to those previously described. The estimated numbers of eubacteria from the frond segments of *A. caroliniana*, *A. filiculoides*, and *A. mexicana* were consistent with the numbers of eubacteria per cavity reported previously for these species (Petro and Gates, 1987). The presence of these organisms in *A. miocrophylla* has not been previously reported. Results of the current study indicate that the eubacteria are present in *A. miocrophylla* in numbers approximately 3-fold greater than we estimated in *A. mexicana* but 20 to 30-fold fewer than estimated for *A. filiculoides*.

Nierzwicki-Bauer and Aulfinger (1991) reported the presence of five ultrastructurally distinct eubacteria in leaf cavities of *A. caroliniana*. Although they attempted to isolate all five types of bacteria observed in micrographs of *A. caroliniana*, only the bacteria resembling *Arthrobacter* were recovered under aerobic conditions on complex media. Thus, the description by these authors of *Arthrobacter*-like organisms as the dominant eubacteria isolated from the leaf cavities of *Azolla* species is in accord with the data presented in this paper and elsewhere (G + C et al., 1988; Petro and Gates, 1987).

All twenty of the eubacteria isolates in this study were similar with respect to cell morphology and physiology. Only one isolate (AM-5) exhibited a different colony appearance after transfer to PCA slants. While the cell morphology of AM-5 is indistinguishable from other isolates, the white, slimy colonies and

diffusible brown pigment were distinctive. The possibility clearly exists that this isolate is not an arthrobacter, but the evidence is currently insufficient to exclude this organism from the genus. Due to the recent report of the isolation of *Agrobacterium* sp. from *A. filiculoides* (Plazinski et al., 1990), it is of interest that AM-5 not only produces extracellular slime in complex media, which is a characteristic of *Agrobacterium*, but also has a mol% G + C (57.7%) similar to that determined in our lab for *Agrobacterium tumefaciens* strain C58 (57.9%).

Within the group of twenty *Azolla*-associated isolates from the four fern species, the DNA base composition varies from a low 57.7% to a high of 68.2% (Fig. 1). Admittedly this range of mol% G + C values is broad; the existence of distinct groups with similar base composition could be interpreted to suggest the presence of different species or even genera. Phenotypically, however, these organisms appear to be members of the same species. All twenty isolates would key to the same organism using classical determininative techniques. This well documented existence of many common phenotypic traits among the isolates, along with the fact that the G + C content is within the range reported for species of *Arthrobacter* argues for the continued placement of the *Azolla* isolates in this genus. The groups of isolates with similar base composition may represent biotypes or strains of the same species with a great deal of genetic diversity.

It must be noted, however, that the taxonomic position of *Arthrobacter* species is controversial (Keedie et al., 1984; Krulwich and Pelliccione, 1979). Although members of the genus all share the characteristic coryneform morphology, they exhibit a wide variety of physiological and metabolic characteristics and a wide range of DNA base composition (Krulwich and Pelliccione, 1979; Skyring and Quadling, 1970; Yamada and Komagata, 1970). Although our data support the taxonomic placement of isolates from each fern species in the genus *Arthrobacter* as it is currently delineated, the mol% G + C data do not rule out the possibility that these organisms could belong to a different genus. DNA base composition data alone are clearly insufficient for taxonomic classification of the *Azolla*-associated eubacteria. Additional studies emphasizing both DNA/DNA and DNA/RNA hybridizations are clearly needed. Such studies should provide a more direct comparison of these organisms and clarify their taxonomic position.

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