

A photocarotenogenic *Rhodococcus* sp. isolated from the symbiotic fern *Azolla*

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Abstract

An aerobic, heterotrophic bacterium, termed strain APG1, was isolated from surface-sterilized fronds of *Azolla pinnata*, a water fern having symbiotic leaf cavities. Based on its fatty acid profile, morphology and biochemical characteristics, strain APG1 was assigned to the genus *Rhodococcus*. Exposure to light caused colonies to yellow due to accumulation of a β -carotene-like pigment. We suggest that photocarotenogenesis and other traits of strain APG1 could facilitate the survival of the bacterium *in planta*.

Introduction

Within dorsal leaf cavities of the aquatic fern *Azolla* are symbiotic communities composed of filamentous N₂-fixing cyanobacteria and heterotrophic bacteria (Peters and Meeks, 1989). The cyanobacteria release ammonia for uptake by the plant host (Meeks, 1998). In turn the plant provides sucrose to the cavity (Kaplan and Peters, 1988; Kaplan and Peters, 1998). Heterotrophic bacteria within the cavities and others closely associated with the plant tissue may benefit the symbiosis via their metabolism of fixed N (Okoronkwo and Van Hove, 1987). Culturable gram-positive bacteria isolated from *Azolla* symbiotic cavities have been identified as *Arthrobacter* species (Caudales et al., 1998; Forni et al., 1990; Wallace and Gates, 1986). Gram-negative isolates from surface-sterilized *Azolla* frond tissue have been identified as *Agrobacterium*, *Alcaligenes*, *Caulobacter* and *Pseudomonas* species (Plazinski et al., 1990; Newton and Herman, 1979).

Here we report the isolation from surface-sterilized *Azolla* fronds of a gram-positive *Rhodococcus* sp. that displays photoinduction of carotenoid formation.

Materials and Methods

Isolation of strain APG1. The source and maintenance conditions for *Azolla pinnata* have been described previously (Cohen et al., 2002b). For surface sterilization, healthy de-rooted fronds were placed in a nylon net and rinsed under a stream of tap water for 30 min. The net with the *Azolla* fronds was then dipped in a 15% Chlorox®/0.01% SDS solution for 1 min, rinsed three times with sterile distilled water, dipped in 3% H₂O₂ for 3 min, and rinsed once with sterile distilled water. To check for surface contamination some fronds were gently blotted and spread across nutrient agar (Difco). Approximately 0.1 g of fronds were ground in a sterile microfuge tube containing 200 µl distilled water. A sample of this homogenate was streaked onto nutrient agar plates and incubated in the dark at 29 °C. After 5 d several small shiny white convex colonies with smooth margins were visible. Upon transfer to room lighting conditions these colonies gradually became yellowish. One of these colonies was streaked onto nutrient agar for isolation and termed strain APG1.

Biochemical and microbiological characterizations. Gram stain reagents were obtained from Ward's Natural Science Establishment, Inc. (Rochester, New York). The KOH test was carried out as described by Wallace and Gates (1986). Assays for starch and gelatin hydrolysis, catalase and oxidase activities, and the requirement for oxygen using nutrient agar "shake tubes," were conducted according to Seeley et al. (1991). To test for utilization of 95 carbon sources, GP2 MicroPlates™ (Biolog Inc., Hayward, CA, USA) were inoculated with strain APG1 and analyzed by the MicroLog™ System 4.0 according to the manufacture's instructions.

Fatty acid and carotenoid analyses. Nutrient agar plates containing 100-500 colonies served as the source of bacteria for fatty acid and carotenoid analysis. The plates had been incubated in darkness or under fluorescent light (60 µmol m⁻² s⁻¹). The colonies were rinsed into two microfuge tubes and a sample removed for OD₆₀₀ determination. The tubes were centrifuged at 10,000 g, the supernatants decanted, the pellets resuspended in distilled water, and again centrifuged and decanted. Fatty acid methyl esters (FAMES) for gas chromatogram analysis were prepared from cells in one of the tubes by saponification, methylation, and extraction as described previously (Cohen et al., 2002a; Meziane and Tsuchiya, 2000). The pellet in the other tube was resuspended in 1 ml of a methanol:acetone (3:1) solution and allowed to sit at room temperature in darkness for 30 min. The tube was then centrifuged at 18,000 g for 10 min and the absorb-

ance spectrum of the carotenoid-containing supernatant measured using a Shimadzu UV-1650PC spectrophotometer.

Results and Discussion

Isolation, morphology and oxygen requirement. Strain APG1 was isolated from an *Azolla pinnata* frond surface sterilized by treatment with bleach and hydrogen peroxide as described in the Materials and Methods. Typical of heterotrophic bacteria previously isolated from *Azolla* (Forni et al., 1990; Plazinski et al., 1990), strain APG1 exhibited slow growth in culture; cells released from the plant took 5 days to form visible colonies on nutrient agar medium. Though not quantified on a per leaf basis, the amount of viable bacteria released by crushing surface-sterilized *A. pinnata* fronds was substantially lower than that from *Azolla filiculoides* (unpublished observation).

Growth of strain APG1 in nutrient agar shake tubes occurred only at the air-medium interface, indicating an obligate requirement for oxygen. In contrast to *Arthrobacter* strains previously isolated from *Azolla* (Forni et al., 1990; Wallace and Gates, 1986), which are phylogenetically gram-positive but most commonly stain gram-negative, strain APG1 consistently stained gram-positive and colonies did not form a viscous suspension when placed in 3% KOH. Cells in culture were catalase positive and oxidase negative, did not hydrolyze gelatin or starch, and were found as rods (up to 4.5 μm long and 0.8 – 1.0 μm diameter) as well as cocci in older cultures.

Fatty acid profile and taxonomic identity. Fatty acid profiles are useful indicators of taxonomic identity. A previous survey of seven bacterial strains isolated from the megasporocarps and leaf cavities from six *Azolla* species found predominantly branched-chain fatty acids (e.g. *anteiso*17:0) present in proportions characteristic of the genus *Arthrobacter* (Caudales et al., 1998), confirming taxonomic designations made based on morphological and biochemical properties (Forni et al., 1990). Strain APG1, however, unlike *Arthrobacter*, has almost no branched-chain fatty acids (Table 1). Based on its fatty acid profile, strain APG1 appears to belong to Cluster I-4 within the taxonomic grouping scheme of coryneform and related gram-positive taxa established by Kapfer and Kroppenstedt (1996). This cluster contains many isolates formerly classified as *Arthrobacter* but which are now assigned to the genus *Rhodococcus* (Collins, 1986; Koch et al., 1995). A MicroLogTM catabolic profile of strain APG1 did not match that of any bacterium in the Biolog database but showed closest similarity to profiles of *Rhodococcus* spp.

Table 1. Fatty acid profiles of strain APG1 cells cultured in darkness and under light.

Fatty acids ^a	Incubation conditions	
	Dark	Light
14:0	3.1 ± 0.1	3.1 ± 0.4
15:0a	0.0 ± 0.0	0.2 ± 0.2
15:0	0.6 ± 0.1	0.2 ± 0.2
16:0	28.7 ± 0.2	25.9 ± 0.4
16:1ω7 <i>cis</i>	4.7 ± 2.1	7.5 ± 3.3
16:1ω7 <i>trans</i>	4.3 ± 2.3	3.5 ± 0.6
17:0	0.4 ± 0.1	0.9 ± 0.0
17:0a	0.0 ± 0.0	0.6 ± 0.1
17:1	0.8 ± 0.5	1.9 ± 0.7
18:0M	4.4 ± 2.2	2.9 ± 0.5
18:1ω7	24.2 ± 3.5	26.6 ± 0.6
18:2ω6	1.0 ± 1.0	0.3 ± 0.3
19:0	8.3 ± 2.8	7.5 ± 1.2
20:0	0.7 ± 0.1	0.3 ± 0.1
20:2	0.5 ± 0.5	0.6 ± 0.4
22:0	6.4 ± 2.0	5.0 ± 0.5
22:2	0.7 ± 0.7	0.9 ± 0.5
24:0	7.2 ± 1.8	6.2 ± 0.4
others	3.2 ± 2.6	3.6 ± 1.0

^aFatty acids are designated as X:YωZ where X is the number of carbon atoms, Y is the number of double bonds and Z the position of the ultimate double bond from the terminal methyl group; a, anteiso; ± range, *n* = 2.

In their survey, K pfer and Kroppenstedt (1996) did not list fatty acids having chain lengths over 20 carbons, perhaps due to the common assumption that longer fatty acids make only negligible contributions to a bacterial fatty acid profile (Arts and Wainman, 1998). We found, however, that >10% of the strain APG1 fatty acids were 22 to 24 C in length (Table 1) and confirmed these sizes by mass spectral analysis (data not shown). Collins (1986) reported 4.3% C_{22:0} in *Rhodococcus erythropolis* (formerly “*Arthrobacter oxamicetus*”) in a table of fatty acid profiles that included chain lengths of up to 22 C.

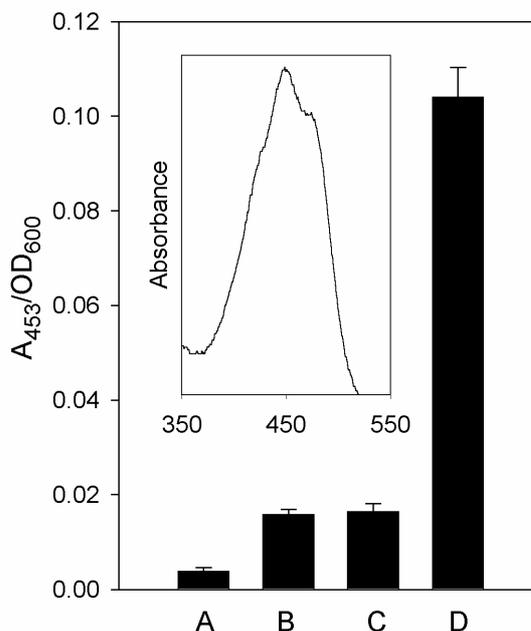


Figure 1: Photocarotenogenesis by strain APG1. A_{453} of methanol:acetone extract per unit of cell density (OD_{600}) for cells cultured on nutrient agar. Cells incubated for 5 d (A), 8 d (B), 10 to 15 d (C) in darkness, and 8 d total with 3 to 5 d under light (D). Values are the mean of at least three independent measurements. Bars, standard error. Inset, absorbance spectrum of extract.

Photocarotenogenesis. Cells of strain APG1 exposed to light accumulated a yellowish-orange pigment at a level about seven times higher than dark-incubated cells (Fig. 1). The absorbance spectrum of the pigment (Fig. 1, inset) was characteristic of β -carotene, a carotenoid common in *Rhodococcus* spp. (Ichiyama et al., 1989) but, to our knowledge, not found in *Arthrobacter* spp. Carotenoid pigments are known to protect cells by physically quenching singlet oxygen formed by photosensitizers such as degradation products of chlorophyll and cytochromes (Tuveson et al., 1988). No significant change in the strain APG1 fatty acid profile occurred in response to light (Table 1).

Conclusions. Based on its fatty acid and carotenoid composition, morphology and biochemical characteristics we have assigned strain APG1 to *Rhodococcus*, a genus not previously reported to have been isolated from *Azolla*. Although strain APG1 was isolated from healthy surface sterilized tissue we cannot conclude that it is part of the *Azolla* cavity community. The bacterium may reside intercellularly within the *Azolla* fronds; pathogenic and non-pathogenic strains of *Rhodococcus fascians* are capable of colonizing the interior of plant leaves without causing disease symptoms (Cornelis et al., 2001) and *A. pinnata* plants that were free of *Rhodococcus* APG1 could be colonized in the laboratory to a stable surface-sterilant resistant frequency of $2-4 \times 10^7$ *Rhodococcus* APG1 cells per gram wet weight of frond tissue (Cohen and Yamasaki, 2003).

Rhodococcus APG1 shows adaptations that appear to be selective for plant-associated survival. Exposure to light induces production of carotenoids, a trait that likely confers some tolerance to the high light irradiance common in the tropical and subtropical habitats of *A. pinnata*. Cells of *Rhodococcus* APG1 cultured on sucrose as a sole C source reach stationary phase at a relatively low density and display an increased tolerance to light and H_2O_2 and a higher per cell nitric oxide synthase (NOS) activity (Cohen and Yamasaki, 2003). NOS activity by *Rhodococcus* APG1 combined with nitrification by associated *Arthrobacter* spp. (Verstraete and Alexander, 1972) could provide nitrogen oxides to *Azolla* plants for regulatory functions (Yamasaki 2000). A method for obtaining large numbers of bacteria from host plants will be necessary for determining if the traits we have investigated are indeed expressed in the plant-associated state.

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