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Lumen Bacteria from Endomycorrhizal Spores

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Abstract. Bacteria were detected in the lumen of endogonaceous chlamydospores of *Glomus macrocarpus* var. *macrocarpus* obtained from the rhizosphere soils of xerophytic plants (*Ficus bengalensis* and *Acacia* spp.) using a scanning electron microscopy technique. Pure cultures of bacteria obtained on synthetic media were identified as species of *Enterobacter*, *Pseudomonas*, and *Streptococcus*; a few unidentified strains were also isolated.

Although much attention has been focused on the form, function, and characteristics of the many vesicular-arbuscular (VA) mycorrhizas that exist in a mutual association with legumes, cereals and other plants [5,16,19], our understanding of microbial inhabitants of endogone spores is meager. Available literature suggests that the surface walls of spores provide a rich environment for the establishment and metabolic maintenance of microbial flora, and fungal and actinomycetous hyphae have frequently been situated on the surface of spores. The porous meshwork system on the wall provides niches where bacteria often grow in abundance and derive energy for their growth. Mosse [15] and Old, Nicolson, and Redhead [21], using transmission electron microscopy, earlier reported the presence of bacteria-like organisms and larger organisms (chytridious fungi, actinomycetes, and protozoans) in the lumens of sessile endogone spores. However, none of these studies have isolated and characterized these bacterial flora that inhabit the lumen of endogone spores.

We report the occurrence of bacteria within endogone spores from the rhizospheres of xerophytic plants as seen by scanning electron microscopy. Bacterial isolates were also grown successfully on synthetic media, and some were identified.

Materials and Methods

Chlamydospores belonging to *Glomus macrocarpus* var. *macrocarpus* were obtained from the rhizospherous soils of typically xerophytic plants (*Ficus bengalensis* and *Acacia* sp.) by a wet sieving and decanting technique [6,27]. The plants had been raised in sterile earthenware pots (14″ diam) and maintained in a greenhouse, and the roots of young seedlings had been inoculated with *G. macrocarpus* var. *macrocarpus* spores [24]. The microbial flora on the surfaces of the recovered spores were killed by exposing the thoroughly washed spores (sterile suspension, 50 mM NaCl) to a germicidal lamp (40 W, Philips) for 30 s, followed by immersing them into 2% (wt/vol) chloramine containing 200 mg streptomycin/liter and a trace of detergent (Triton-100; 0.12%) plus 0.01% dodecyl sodium sulphate [17,28].

Bacterial cells were extracted by macerating the surface-sterilized spores in a glass homogenizer (Bachofer, 4 ml Duran 50) containing 50 mM saline solution and small quantities of acid-washed fine quartz sand [10,22]. The homogenate was clarified by centrifuging at 1,500 × *g* for 8 min at 5°C (Sorvall, RC 5). The supernatant was routinely used to make serial dilutions up to 10^{-6} [26]. Tubes filled with medium were then secured horizontally in an aquatherm water bath shaker [11] operating at 260 rpm. Isolates were obtained from 10^{-6} dilution tubes by streaking roll tubes, and they were considered pure cultures after successive passage in roll tubes.

The enrichment medium consisted of equal portions of two solutions. Solution I contained (g/liter distilled water): glucose, ribose, glycerol, and mannitol, 8.0 each; $MgSO_4 \cdot 5H_2O$, 1.0, NaCl, 0.02; $FeSO_4 \cdot 7H_2O$, 0.3; $Na_2MoO_4 \cdot 2H_2O$, 1.3; sodium thioglycolate, 1.0; and resazurin, 0.002. Solution II contained 1% (wt/vol) of a vitamin solution in 0.1 M potassium phosphate buffer (pH 7.2). In addition, MB medium was prepared as roll tubes with 100% CO_2 as the gas phase and were stored in anaerobic screw-cap culture tubes [8]. The composition of MB medium was described by Bryant [1] and was used here with 0.75% glucose, maltose, and cellobiose as the carbohydrate sources. Aerobic nutrient broth contained (g/liter): peptone, 10; meat extract, 10; and NaCl, 5. The pH was adjusted to 7.4. Solid media contained 1.5% agar.

Growth studies were conducted essentially using basal medium. Its composition was similar to that of the enrichment medium, except that ribose, glycerol, mannitol, thioglycolate, and resazurin were omitted and the glucose concentration was increased to 1.4%. Gram staining was performed with Kopeloff reagents. The motility of the cells was determined by direct observation of wet mounts of broth culture using phase-contrast microscopy. The number of cultivable bacteria per spore was calculated from the average number of colonies present on primary isolation plates or in roll tubes and also by using the acridine-epifluorescence method [3]. Isolates were characterized

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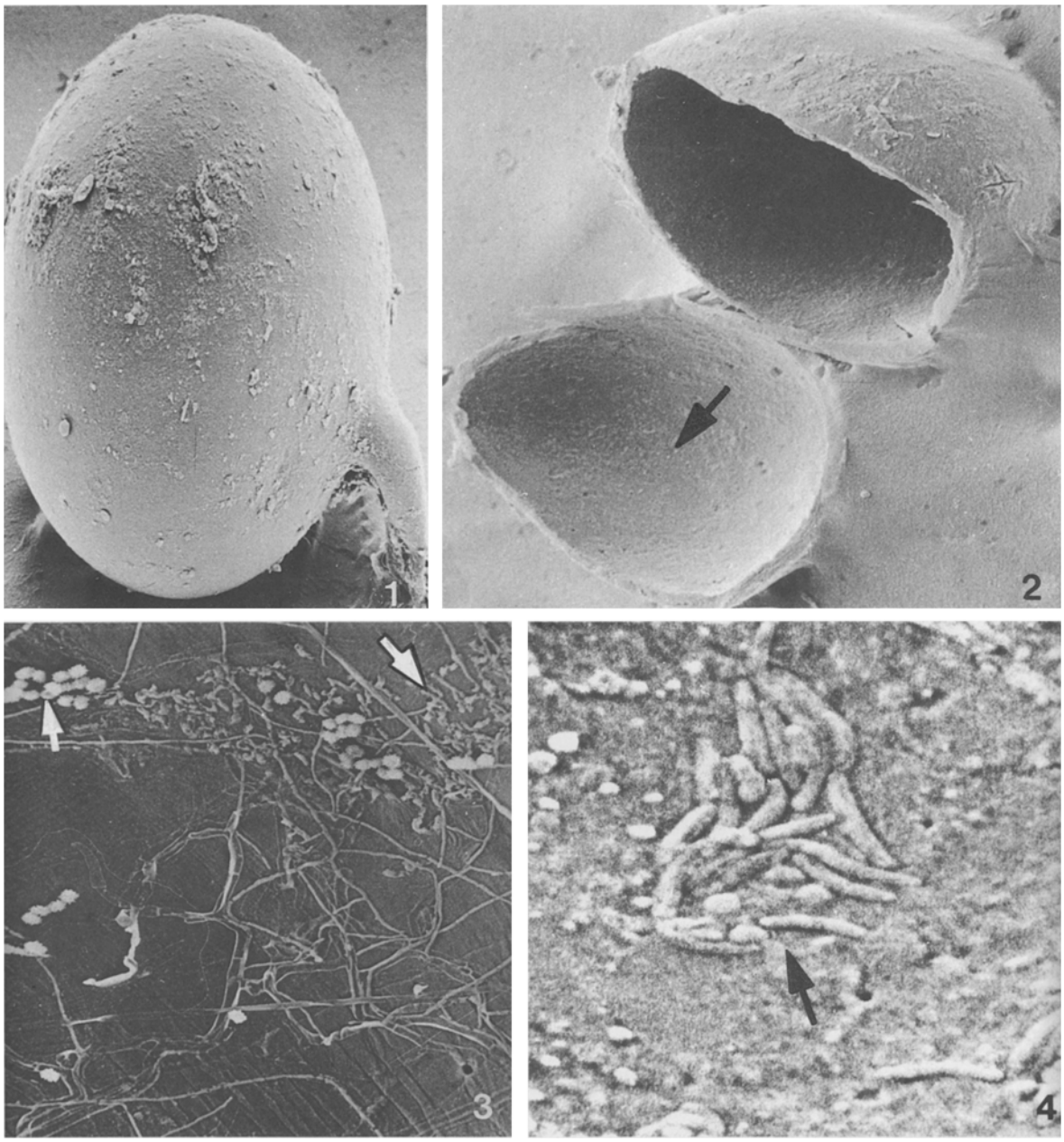

Figs. 1–4. Scanning electron micrographs of *Glomus macrocarpus* var. *macrocarpus* with wall layers and attachment. Fig. 1. Whole spore, showing its outer surface and hyphal attachment (×560). Fig. 2. Whole spore, split in two. The arrow indicates the presence of bacteria (×540). Fig. 3. Inner membrane of the spore. Note the bacterial colonies, in which rod-shaped and coccoid cells (arrow) are seen. Fungal and actinomycetes hyphae are also seen on this membrane (×1,800). Fig. 4. Inner surface of the spore, showing clusters of bacterial cells (×4,400).

by standard microbiological methods [2]. A variety of known bacteria were used as positive and negative controls.

Sugar fermentation reactions were evaluated by using phenol red broth base (Difco) in which 1% test sugar was included [7,29]. Sugars were sterilized separately as 1% solutions, except esculin, which was sterilized with broth base. KCN sensitivity was determined as described by Holding and Collee [9] and omitting thioglycolate. Nitrate reduction was tested following standard procedures [13], and G+C content was determined using the density method [23].

Scanning electron microscopy (Cambridge Stereo Scanner, model S4-10) was done by dehydrating (graded series of ethanol) the well-washed spores (graded washing in 50–250 mM saline solution) and transferring them onto an I.C. aluminum stub (10 mm diam), with the help of a needle, coated with pressure-sensitive adhesive tape. Samples were coated in an Edwards

Table 1. Bacteria isolated from lumen of endogone spores.[a]

Origin of isolates and development stages of plants		Viable bacteria per spore ($\times 10^{-4}$)	Number of isolates belonging to:				Total no. of isolates characterized
			Enteric type *(Enterobacter)*	*Pseudomonas* spp.	*Streptococcus* spp.	Unidentified rods	
Ficus bengalensis	(a)	2.4	7	3	1	0	11
	(b)	2.9	8	3	2	1	14
	(c)	2.8	11	6	2	2	21
Acacia sp.	(a)	1.9	4	4	2	2	12
	(b)	2.1	4	5	2	3	14
	(c)	2.5	6	4	2	4	16

[a] The symbols a, b, and c represent 6-, 12-, and 18-week-old plants, respectively. Data are average of five experiments. Enteric type is assigned to the family Enterobacteriaceae. Growth studies were performed with standard tests as described in Material and Methods.

vacuum evaporation unit at a pressure of 10^{-15} torr with approximately 1.0-pm-thick layer of carbon followed by 2.0-pm-thick coating of silver. Scanning was performed at a tilt of 45° and an anode potential of 100 kV [12]. All chemicals were of analytical-reagent grade and were obtained either from Sigma Chemical Co., Louis, Missouri, USA, or from BDH, Poole, United Kingdom.

Results

A whole spore at low magnification is shown in Fig. 1. When the spore was crushed into two or three portions, the inner membrane was visible (Fig. 2). Bacterial cells on the inner surface were more clearly visible at a high magnification (Fig. 3). Randomly distributed rod-shaped or coccoid cells were clearly seen, and cells undergoing binary fissions were evident (Fig. 3, arrows). The inner membranes were also colonized by fungal and/or actinomycete hyphae (Fig. 3). Figure 4 shows clusters of bacterial rods and coccoid cells at a higher magnification.

Thirty-three endogone spores selected from the rhizospheres of *Ficus bengalensis* and *Acacia* sp. at different growth stages were screened; with one exception, all contained bacterial cells. An enumeration of bacterial isolates is given in Table 1. Not many differences in bacterial flora were seen between the spores of *F. bengalensis* and *Acacia* sp. Enteric-type bacteria were always obtained and were the predominant cultivable organisms, accounting for about 45% (40/88) of all isolates. *Pseudomonas* spp. followed next (28%) and then *Streptococcus* spp. (12%). Unidentified isolates constituted a minority. Bacteria were identified with the aid of Bergey's Manual of Determinative Bacteriology. Spores at different ages of plants represented a similar bacterial system. During the initial phase of this study, a variety of isolation media were screened for their suitability. Media described here were the most suitable in terms of the number of viable cells per spore or the nature of isolates and therefore were chosen for routine use because they permitted rapid and profuse colony development and were rapidly prepared.

Two controls were run to test the presence of any bacteria on the surface of the spore wall after the surface sterilization treatment: (1), when surface washes of surface sterilized spores were taken as inocula, colony counts were much lower and different bacteria were isolated; (2), the treated spores (74) were seeded on several defined solid and liquid media and incubated up to 72 h (28°C and 37°C ± 2). On edges of only 3 spores, a few bacterial colonies were observed, mixed with hyphal growth (probably actinomycete and fungi). These controls indicated that bacterial colonies described in Table 1 were not spore-wall-surface contaminants but were true inhabitants of the lumen of endogone spores.

Separate controls were also run in order to determine whether endogone spores under study were viable or moribund. The viability of the mycorrhizal spores was tested by repeatedly transferring them onto newly grown seedlings under strict aseptic conditions. In all tests, the spores infected the host root and produced vesicles and arbuscles.

The general properties of bacterial isolates are presented in Table 2. Isolates of *Pseudomonas* spp. were characterized by fluorescent pigments in ultraviolet light, and arginine dihydrolase was absent. These cells were not able to hydrolyze starch, gelatine, or poly-β-hydroxybutyrate. The optimum

Table 2. Salient properties of bacteria isolated from endogone spores.

Isolates	Morphology	Gram reaction[a]	Dimension μm	Motility	Relation to oxygen	Catalase reaction[a]	G+C content of DNA (moles %)
Enteric Type (*Enterobacter* sp.)		−	0.4–1 by 1.4–3.8	− or +	Facultative	+	53.6 –56.4
Pseudomonas sp.		−	1.2–1.6	+	Strict aerobe	+	61–68
Streptococcus sp.		+	0.8–1.5	−	Facultative	−	36–38
Unidentified spp.		− and +	variable	+ or −	Anaerobe	−	Not determined

[a] The signs + and − indicate positive and negative reactions in the indicated procedures.

growth temperature was 26°C ± 1. They required no growth factors and were able to grow in a purely mineral medium with the addition of glucose or acetate (sodium, ammonium, and potassium salts, in order of preference).

Enteric-type colonies on nutrient agar were smooth, had a shining surface, and grew well on glucose as the carbon source. Cells were peritrichously flagellated. The optimum temperature for growth was 37°C ± 1. Biochemical reactions are presented in Table 3. Sugar fermentation tests indicated that the isolates fermented glucose, sucrose, lactose, maltose, xylose, trehalose, inositol, esculin, arabinose, and dulcitol.

Table 3. Characterization of enteric-type bacterial isolates from spores of *Glomus macrocarpus* var. *macrocarpus*.

Substrate	Reaction
TSI slant	Acid
TSI base	Acid
TSI gas	+
TSI H_2S	−
Methyl red (37°C)	+
Voges-Proskauer (37°C)	+
Citrate (Simmons)	+
Indole	−
KCN	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
Arginine dihydrogenase	−
Urease	−
Jordan's tartrate	−
Nitrate to nitrite	+
Oxidation-fermentation	F
Oxidase	−

[a] The following symbols are used: +, Positive reaction; − negative reaction; F, fermentation. TSI, triple sugar iron agar (Difco). Products were determined after growth had ceased. The experiments were carried out at 37°C in 100-ml closed culture bottles containing 50 ml medium.

Discussion

There is now increasing interest in endomycorrhizas of plants growing under arid conditions [4,14,18,20], because endomycorrhizas may be of considerable significance in the ecology of plants growing under stress [16,28]. We earlier recorded the occurrence of endogonaceous spores from the Aravalli hills in northern India: These hills are a prominent feature in the old palaeozoic and mesozoic geography in the Indian subcontinent, and the plant communities form a dry deciduous-shrub forest [25]. Aravalli rocks include numerous secondary aluminous and calcareous silicates and laccolites, lodes of copper, lead, and zinc, and traces of nickel and cobalt. Mosse [15] reported the bacteria-like organisms that may be actinomycete spores living symbiotically from sessile endogone spores and multiplying greatly as the spore enters dormancy. Mosse [15] further emphasized that the metabolic products from rapidly multiplying actinomycete spores would then be available for the host (endogone spore). Old, Nicolson, and Redhead [21] have made a similar observation concerning the lumen of honey-colored sessile endogone spores from Nigeria. However, they did not regard their microbial flora as true, natural, symbiotic-like organisms.

Bacteria grow in the lumen of spores occurring in such arid areas, both naturally and in association with experimental plants maintained in a greenhouse. The physiological significance of such associations needs careful study.

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