Characterization of a New Anastomosis Group (AG-9) of *Rhizoctonia solani*

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ABSTRACT

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Isolates of *Rhizoctonia solani*, anastomosis group -9 (AG-9), fruited on 2% V-8 juice agar. Metabasidia were barrel shaped, not constricted about the middle, 11.6–22.3 × 6.9–10.8 μm, and somewhat wider than supporting hyphae. Sterigmata were 9.2–63.9 μm long and numbered 1–5 per basidium. Adventitious septation in sterigmata was rare. Basidiospores were 7.7–11.6 × 4.6–7.7 μm, smooth, thin walled, hyaline, and prominently apiculate. Spores tended to be unilaterally flattened and widest at the distal end. Repetition of basidiospores was not observed. Morphological characteristics of the perfect state indicate that isolates of AG-9 are properly classified as *Thanatephorus cucumeris*. Mycelium of isolates of AG-9 growing on potato-dextrose agar was whitish to light tan initially, becoming dark brown with age. Sclerotia, generally lighter in color than the mature mycelium, were scattered randomly over the agar surface. Concentric rings were produced by most isolates. In a growth chamber study, isolates of AG-9 were not pathogenic on established plants representing nine species. However, field recovery of isolates of AG-9 from root lesions on lettuce and carrot and from lesions on the subterranean and aerial stems of potato indicate that they may be mildly parasitic. Most isolates of AG-9 were thiamine-autotrophic, but some were auxotrophic. Thus, AG-9 differs from other anastomosis groups of *R. solani*, where thiamine requirement is reported to be a groupwide characteristic.

Rhizoctonia solani Kühn [*Thanatephorus cucumeris* (Frank) Donk] is a plant parasitic fungus commonly observed in association with potato and vegetable crops grown in Alaska (2, 3). Over the past several seasons, isolates of *R. solani* that do not fuse with tester isolates representing any of the reported anastomosis groups (AG) have been recovered from soil and plant samples collected in south central and interior Alaska. A similar isolate was collected near Pendleton, OR. An earlier report (3), wherein it was proposed that this group of isolates be designated AG-9, documented some characteristics of the imperfect stage of these isolates, including pathogenicity on sprouting potatoes and germinating cauliflower seeds. This report contains additional information on mycelial characteristics, thiamine requirements, and a description of the perfect stage.

MATERIALS AND METHODS

Collection and isolation. Isolates of *R. solani* were collected from soil and from plant tissue. Indirect and direct methods were used to isolate from soil. When the indirect method was used, a 50-g sample of soil was placed in a 10-cm-diameter petri dish with 0.5 g (approximately 50 seeds) of autoclaved beet (*Beta vulgaris* L.) seeds (16). Samples were incubated for 72 hr at 21 C, and then the contents of each dish were emptied into a 2-mm mesh sieve and rinsed with cold running tap water for 2 min. Washed beet seeds (9–10 seeds per dish) were placed in petri dishes containing 2% water agar supplemented with 50 mg/L of streptomycin sulfate and chlorotetracycline hydrochloride. After a 24-hr incubation at room temperature, hyphae with characteristics similar to those of *R. solani* growing from the beet seeds were excised and placed in petri dishes containing rehydrated potato-dextrose agar (PDA). Following transfer to 2% water agar to eliminate fungal and bacterial contaminants, isolates were placed on PDA slants and stored at room temperature.

When isolating directly from soil, a soil pellet technique (6) was used with the Ko-Hora medium (8) amended with prochloraz (5 μl/L) (Boots Chemical Co., Nottingham, England) (4). Prior to pelletizing and plating of soil, 5 ml of a 1,000-μl/L aqueous solution of prochloraz was atomized into a 95-g sample of soil. Following a 1- to 2-hr incubation period, soil pellets were placed onto the Ko-Hora plus prochloraz (KHP) medium using the soil pelleting mechanism described by Henis et al (6). After a 3-day incubation at room temperature, hyphae with characteristics similar to those of *R. solani* growing from the soil pellets were isolated, transferred, and stored as described above.

Isolates from plants were collected in one of two ways. Small pieces of lesioned root or stolon tissue were excised, surface-disinfected in 1.0% sodium hypochlorite for 30 sec, rinsed, and then placed on PDA. Small pieces of hymenia were excised from potato stems and then placed directly in petri dishes containing PDA. The surface-disinfection step was bypassed with hymenial isolations, due to the delicate and superficial nature of hymenia. Transfer and storage of isolates collected from plants was as described for isolates from soil.

Anastomosis group typing. Anastomosis group identities were determined according to the modified (4, 2) method of Parmeter et al (17). Isolates were paired on 3 × 1.5-cm rectangles of cellophane placed on 1.5% water agar in petri dishes. Cellophane rectangles had been dipped in soft (13 g/L) PDA before being placed on water agar. A mycelial disk from a tester isolate was placed on one end of the cellophane rectangle and a disk from a field isolate was placed on the other. Mycelial disks of tester and field isolates were produced on PDA. Anastomosis plates were incubated at room temperature until hyphae overlapped, usually 48–72 hr. The area of cellophane upon which hyphae overlapped was then removed from the agar, placed on a slide, stained with 0.05% trypan blue in lactophenol, and examined microscopically (400×) for hyphal anastomosis. The fusion of cell wall and plasmalemma accompanied by death of anastomosing and adjoining cells (K reaction) (5,14) was sought, and at least five fusion sites were required for each positive anastomosis reading.

Field isolates of *R. solani* AG-9 selected as tester isolates were paired on anastomosis plates with two tester isolates representing AG-1, AG-2-1, AG-2-2, AG-3, AG-4, AG-5, AG-6, AG-7, AG-8, and AG-BI (3). Other isolates suspected of being *R. solani* AG-9 were paired with established AG-9 testers to confirm their affiliation with AG-9.

Cultural characteristics. Isolates of *R. solani* AG-9 were grown on PDA in the dark at room temperature. Observations were made over a 3-wk period.

Hyphal diameter. Mature hyphae from the edge of active cultures of *R. solani* AG-9 growing on PDA were placed on slides, stained with 0.05% trypan blue in lactophenol, and observed

microscopically at 400×. Twenty-five observations were made per isolate.

Nuclear number. Mature hyphae from active cultures of *R. solani* AG-9 growing on PDA were placed on slides, stained with Safranin-0 (1), and observed microscopically at 400×. Fifty observations were made per isolate.

Thiamine requirement. Petri dishes containing Czapek-Dox agar with or without 10^{-5} M thiamine hydrochloride (18) were seeded with 2-mm-diameter mycelial disks of isolates representing AG-3 (thiamine-auxotrophic), AG-5 (thiamine-auxotrophic), and AG-9. Colonial morphology and color were observed and mycelial dry weight determinations were made after 2 wk of growth at room temperature in the dark.

Radial growth. Petri dishes containing PDA were seeded with 4-mm-diameter mycelial disks taken from the edge of actively growing cultures of *R. solani* AG-9. Thirty-eight isolates were included in this study. Cultures were maintained at 21 C and radial growth was measured once daily until the colony reached the edge of the dish.

Pathogenicity determination. Pathogenicity was determined in a growth chamber on barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.), carrot (*Daucus carota* L.), radish (*Raphanus sativus* L.), bean (*Phaseolus vulgaris* L.), tomato (*Lycopersicon esculentum* Mill.), eggplant (*Solanum melongena* L.), cauliflower (*Brassica oleracea* L.), and potato (*Solanum tuberosum* L.). Plants were maintained under 12-hr fluorescent light at temperatures of 25–32 C. Dark period temperatures were 16–25 C. Plants were grown in 2,000-ml plastic pots containing a 2:1 sand/soil mixture. The soil mixture was infested 4 wk after planting by placing 10 barley kernels colonized by isolates of AG-9 1 cm below the soil surface in each pot (one kernel/200 ml soil). Plants were harvested 10 wk after infestation, when root systems were washed and examined for necrotic lesions. An attempt was made to isolate *R. solani* from the root systems of each plant species.

Perfect stage development. Attempts were made to induce the perfect stage of *R. solani* AG-9 to form in 2% water agar, V-8 juice agar (18 g/L agar plus 20 ml/L V-8 juice), and with the soil overlay technique (12). These studies were made at room temperature with diurnal room light.

RESULTS

Collection and anastomosis grouping. A total of 74 isolates of *R. solani* AG-9 were collected over a 4-yr period (Table 1). Most (57) were collected from potato field soil; 11 were isolated from potato plants. Two isolates were collected from other plants (carrot and lettuce), and the remainder were collected from soil associated with small grain or grasses.

Six isolates of AG-9 were selected as potential tester isolates and were paired with two tester isolates representing each of the 10 reported anastomosis groups (3). Selections were made based on collection location and plant association. None of the six isolates anastomosed with tester isolates representing the other 10 AG, but each anastomosed [anastomosis accompanied by cell death (K reaction)] with the other isolates of AG-9. Each of the six isolates anastomosed without cell death (S reaction) when paired with itself on anastomosis plates. Each of the other isolates categorized as a member of AG-9 anastomosed when paired with tester isolates of AG-9.

Cultural characteristics. Isolates of AG-9 growing on PDA initially were whitish to light tan in color, but after 3 wk most isolates had become dark brown. Medium- to large-sized sclerotia were produced by most isolates, although some isolates produced very few. Sclerotia generally were a lighter shade of brown than the mycelium and were scattered over the agar surface. Mycelium generally was appressed to the agar surface, but an occasional isolate produced floccose mycelium. Zonation in the form of concentric rings was present at some stage of growth in nearly all isolates of AG-9 growing on PDA. On Czapek-Dox medium amended with thiamine hydrochloride, zonation was not obvious and colonial color tended to be a lighter shade of brown.

Hyphal diameter. Diameters of individual mature hyphae of the 10 isolates examined ranged from 4.62 to 9.24 μm, and mean diameters ranged from 6.33 to 7.53 μm.

Nuclear number. The mean number of nuclei in mature cells of the nine isolates examined ranged from 5.24 to 7.92 (Table 2). Most cells contained 4–9, but the combined range was 3–16 nuclei per cell.

Thiamine requirement. Isolates of AG-3 grew at approximately the same rate on Czapek-Dox medium whether or not thiamine was added (Table 3). Isolates of AG-5 grew sparsely on Czapek-Dox, but luxuriant growth was observed when the medium was amended with thiamine. Most isolates of AG-9 grew at a comparable rate on Czapek-Dox with and without added thiamine. The growth of two isolates (S9R1 and 86-1) was comparatively slow on the thiamine-minus medium, which indicates a thiamine requirement. An analysis of variance on this nested model with all terms treated as fixed effects indicated that the difference between autotrophs (isolates with B/A ratios of 1.5 or less) and auxotrophs (isolates with B/A ratios of 3.2 or greater) (Table 3) was significant ($P = 0.05$).

Radial growth. Radial growth of the most rapidly growing isolate (S1R2) reached the edge of a petri dish in 3.5 days. Twenty-nine of the 38 isolates had reached the edge within 4 days, whereas the slowest isolate (S2R1) required 9 days to reach the edge of the dish.

TABLE 1. *Rhizoctonia solani* AG-9 isolate collection summary

Collection site[a]	Collection date	No. of isolates	Source of isolates	Remarks[b]
Palmer 1	1983	1	Soil	Ko-Hora plus prochloraz (KHP)
Palmer 2	1983	13	Soil	KHP
Palmer 3	1983	2	Soil	KHP
Palmer 4	1984	1	Potato	Hymenium on stem
Palmer 5	1984	1	Potato	Hymenium on stem
Palmer 6	1984	11	Soil	Beet seed bait
Palmer 7	1984	4	Potato	Hymenium on stem
		2	Potato	Lesion on stolon
Palmer 8	1986	1	Lettuce	Lesion on root
Palmer 9	1986	12	Soil	10 KHP, 2 beet seed
		1	Carrot	Lesion on root
		1	Potato	Hymenium on stem
		1	Potato	Lesion on stem
Palmer 10	1986	18	Soil	6 KHP, 12 beet seed
		1	Potato	Hymenium on stem
Delta 1	1984	1	Soil	Beet seed bait
Delta 2	1984	2	Soil	Beet seed bait
Pendleton	1986	1	Soil	Oat straw bait

[a] Palmer sites located in south central Alaska in the vicinity of Palmer. Delta sites located near Delta Junction in interior Alaska. Pendleton site located near Pendleton, OR.

[b] Palmer and Delta isolates collected from soil were recovered directly on KHP medium or indirectly from sterilized beet seed baits incubated for 3 days in respective soils. The Pendleton isolate was collected indirectly from sterilized oat straw incubated in soil collected from a nonagricultural field by Dr. A. Ogoshi.

TABLE 2. Number of nuclei in hyphal cells of *Rhizoctonia solani* AG-9[a]

	No. of nuclei per cell		
Isolate no.	Mean	Range	Standard error
V12M	5.2	3–8	0.15
S9R1	5.4	4–8	0.13
DEL2	7.5	5–13	0.24
F57M	5.3	3–8	0.16
S14C2	5.9	4–9	0.17
S4B2	7.9	5–16	0.28
S21[b]	6.1	4–8	0.15
S7R1	6.0	4–8	0.14
F33M	6.3	4–9	0.17

[a] Fifty observations per isolate.

[b] S21 = ATCC 62804.

Pathogenicity determinations. No evidence of necrotic lesions or other disease symptoms were observed on eight of the nine plant species evaluated. A small necrotic lesion (<1 mm long) was observed on a tomato root, but *R. solani* was not recovered from the one small lesion or from the roots of any of the nine test species.

Perfect stage development. Isolates of AG-9 did not form a perfect stage when a 2% water agar medium or the soil overlay technique was used. However, many isolates, of which five are reported, were induced to form hymenia and sporulate on V-8 juice agar. Basidia with sterigmata and spores are illustrated in Figure 1.

Hymenia began forming in small, sparse tufts from 1 to 3 mm in diameter on the V-8 juice agar surface. Hymenia began to appear approximately 2 wk after seeding, and basidiospores within 4 wk. Metabasidia were barrel shaped to subcylindrical, not constricted about the middle, and somewhat wider than the supporting hyphae (Table 4). Sterigmata were straight, usually longer than the basidia when mature, and varied in number from one to five per basidium (Table 4). Adventitious septation in sterigmata was rare. Basidiospores were smooth, hyaline, thin walled, and prominently apiculate. Basidiospores tended to be oblong and unilaterally flattened, with the distal end often being widest. Repetition of basidiospores was not observed among the sporulating isolates of AG-9.

With the exception of the length of sterigmata, dimensions of perfect stage structures fell within the range reported for isolates in other anastomosis groups of *R. solani* (Table 5). Sterigmata of most isolates exceeded the reported range for *T. cucumeris* (Table 4), and the sterigmata of one isolate (S7R1) averaged nearly twice the reported range maximum.

DISCUSSION

Most of the isolates of *R. solani* AG-9 collected to date have been found in association with the potato plant or soil in which potatoes grew. This suggests a relationship between AG-9 and potato plants that may not be real. All of the isolates of AG-9 have been collected as part of other studies, and most of these studies have involved potatoes and fields with a history of potato production. Given these circumstances, most isolates obviously would be associated with potato. Isolates of AG-9 have been recovered from carrot and lettuce root and from soil associated with grain and forage grasses. We believe that further surveys may reveal the presence of AG-9 in a variety of environments (3).

Thiamine requirement is reported to be a characteristic of anastomosis groups rather than a characteristic of individual isolates (13,18), and it has been used as a diagnostic tool to distinguish between members of certain closely related anastomosis groups. Isolates of AG-3 are thiamine-autotrophic; therefore, growth rates are similar on Czapek-Dox media with or without supplemental thiamine hydrochloride (Table 3). Isolates of AG-5, on the other hand, are thiamine-auxotrophic, and growth is sparse unless thiamine is added to the medium (Table 3). Growth rates of most isolates of AG-9 (Table 3) suggest that AG-9 is a thiamine-autotrophic group. However, two isolates (SR91 and 86-1) grew poorly on the unamended Czapek-Dox medium, indicating that these and perhaps other isolates are auxotrophic for thiamine. The general implication of variability in thiamine requirement among isolates in this anastomosis group is not clear.

TABLE 3. Thiamine requirement of isolates of *Rhizoctonia solani* representing anastomosis groups 3, 5, and 9

Anastomosis group	Isolate no.	Source	Mycelial dry weight (mg)[a] Czapek-Dox (A)	Czapek-Dox + T[b] (B)	B/A
AG-3	W14L[c]	Alaska	184	216	1.2
	R-542	Japan	235	227	1.0
AG-5	ST-6-1	Japan	8	269	31.8
	T-441		6	273	42.8
	GM-10	Japan	19	255	13.2
AG-9	S21[c]	Alaska	187	204	1.1
	S4B2	Alaska	167	181	1.1
	S1C2	Alaska	148	214	1.4
	F57M	Alaska	50	73	1.5
	DP27	Alaska	202	213	1.1
	M6	Alaska	221	220	1.0
	MC35b3	Oregon	214	212	1.0
	S9R1	Alaska	18	209	11.5
	86-1	Alaska	61	195	3.2

[a] Mycelial dry weight figures are the average of at least three replications.
[b] Czapek-Dox medium ammended with 10^{-5} M thiamine hydrochloride.
[c] W14L = ATCC 62803, S21 = ATCC 62804.

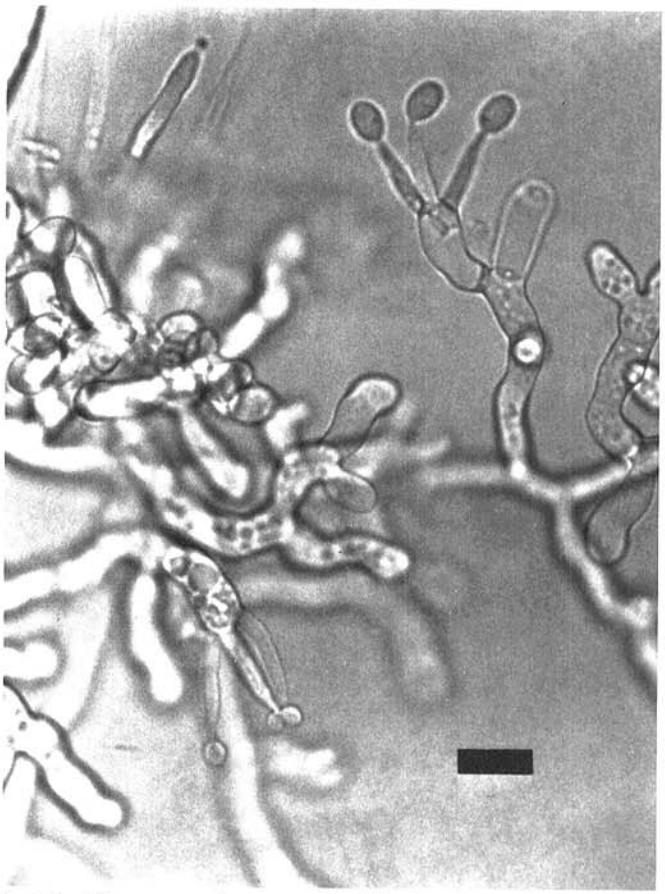

Fig. 1. Basidiospores, sterigmata, and basidia of an isolate of *Rhizoctonia solani* AG-9. Bar = 20 μm.

TABLE 4. Dimensions of sexual structures of *Thanatephorus cucumeris* isolates produced by *Rhizoctonia solani* AG-9[a]

Isolate no.	Metabasidia Length	Metabasidia Width	Sterigmata No./basidium	Sterigmata Length	Basidiospores Length	Basidiospores Width
V12M	14.9 ± 2.1[b]	9.0 ± 1.0	3.9 ± 0.5	29.0 ± 7.3	9.2 ± 0.9	5.7 ± 0.7
	11.6–19.2[c]	7.7–10.8	3–5	16.9–46.2	7.7–10.8	4.6–6.9
F33M	15.1 ± 1.8	8.3 ± 0.7	3.6 ± 0.7	23.4 ± 6.1	9.0 ± 1.2	6.1 ± 0.8
	11.6–18.5	7.7–10.0	2–4	14.6–34.7	7.7–11.6	4.6–7.7
S21[d]	17.3 ± 1.8	8.6 ± 0.8	3.8 ± 0.6	33.3 ± 7.2	8.7 ± 0.7	5.7 ± 0.6
	13.9–20.0	6.9–10.0	2–5	18.5–51.6	7.7–10.0	4.6–6.9
DEL1	17.2 ± 2.3	9.0 ± 0.7	3.7 ± 0.9	21.6 ± 5.4	9.4 ± 0.9	5.9 ± 0.8
	13.9–22.3	7.7–10.8	1–5	9.2–31.6	7.7–10.8	4.6–6.9
S7R1	16.6 ± 2.3	8.2 ± 0.5	3.5 ± 0.6	36.1 ± 12.5	9.1 ± 0.9	5.6 ± 0.6
	11.6–20.0	7.7–9.2	2–4	18.5–63.9	7.7–10.8	4.6–6.9

[a] Dimensional measurements, expressed in μm, are based on 20 observations per isolate.
[b] Mean ± standard deviation.
[c] Range.
[d] S21 = ATCC 62804.

Thiamine requirement may not be used as a diagnostic characteristic for AG-9.

The descriptive information on sexual structures produced by isolates of AG-9 (Table 4) generally is consistent with descriptions for other anastomosis groups of *R. solani* (Table 5); thus, isolates of AG-9 appear to be properly placed as an additional anastomosis group within the species *T. cucumeris* (Frank) Donk. We believe that the excessive length of sterigmata produced by several isolates (Table 4) is due to factors related to the environment in and the method by which the perfect stage was produced. Isolates of AG-1 that we have induced to sporulate have produced sterigmata exceeding the reported maximum length, and it is possible that representatives of other anastomosis groups would also produce longer sterigmata if they were induced to fruit under similar conditions.

Isolates of AG-9 did not damage the root systems of established plants of the nine species evaluated herein, nor could the fungus be recovered from root systems of these plants. However, field isolations of AG-9 have been made from lesions on potato stems, carrot roots, and lettuce roots. Although the time of lesion development and the precise role of isolates of AG-9 in lesion formation under field conditions is unknown, the possibility of a mild pathogenesis in the field on established plants representing several genera is indicated. In addition, small lesions induced by isolates of AG-9 were observed on potato sprouts, and hypocotyl necrosis was observed on in vitro-germinated cauliflower seedlings (2). The data on cauliflower seedlings and sprouting potatoes (2) indicate that isolates of AG-9 may be destructive to the early seedling stages of some plants, but they have a limited capacity to damage the tissues of established plants.

Isolates of AG-9 can damage cauliflower seedlings and possibly seedlings of related and other plant species. Nevertheless, they do not appear to be aggressive plant pathogens. In terms of pathogenicity, isolates of AG-9 may be similar to isolates of AG-6, a generally nonpathogenic anastomosis group that occurs in noncultivated soils in Japan. A type isolate of AG-9 (ATCC 62804) is on file at the American Type Culture Collection in Beltsville, MD.

TABLE 5. Comparison of sexual structures of *Thanatephorus cucumeris* among anastomosis groups of *Rhizoctonia solani*[a]

	Basidia		Sterigmata		Spores	
AG[b]	Length	Width	No./ basidium	Length	Length	Width
1	10.1–20.0	7.2–11.3	(2–5)	3.1–17.2	5.4–11.8	3.9–7.2
2-1	8.6–23.3	4.9–10.7	(1–5)	4.9–18.4	6.0–9.7	3.4–6.8
2-2	7.8–20.4	5.9–11.8	(1–5)	4.9–34.9	5.8–13.1	2.9–6.9
3	7.8–20.4	5.9–11.8	(1–5)	4.9–19.6	6.8–11.3	3.9–7.3
4	6.9–21.6	6.9–11.8	(1–4)	4.9–25.5	5.9–12.7	3.4–7.4
5	6.8–17.5	5.8–10.8	(2–4)	4.9–23.5	3.9–9.8	2.9–6.4
6	7.5–17.0	5.5–10.0	(3–5)	4.5–18.8	5.0–10.0	2.8–5.2
7	11.0–27.5	6.5–11.0	(1–4)	5.0–18.0	6.5–13.0	4.0–8.5
BI	8.8–19.0	7.0–11.3	(3–5)	4.0–12.0	5.0–10.0	3.7–6.7
8	...	...	...	...	...	...
Range[c]	6.8–27.5	5.5–11.8	(1–5)	3.1–34.9	3.9–13.1	2.8–8.5
9[d]	11.6–22.3	6.9–10.8	3.7 (1–5)	9.2–63.9	7.7–11.6	4.6–7.7

[a] Structural dimensions in μm.

[b] Data on AG-1 reported by Oniki et al (15); AG-2-1, AG-2-2, AG-3, and AG-4 by Ogoshi (11); AG-5 by Ogoshi (10); AG-6 and AG-BI by Kuninaga et al (8); AG-7 by Homma et al (7). Data for AG-8 (9,17) are not yet available.

[c] Range established from above listed data for AG-1, AG-2-1, AG-2-2, AG-3, AG-4, AG-5, AG-6, AG-7, and AG-BI.

[d] Range of dimensions for the five AG-9 isolates presented in Table 4.

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