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Release of Sporangiospores by a Strain of *Actinoplanes*

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Dehiscence of *Actinoplanes* sp. 7-10 sporangia is triggered by wetting of the spores. This process requires time because of the hydrophobic nature of the sporangial envelope; it can be speeded up and enhanced by a wetting agent. Once wetted, the spores swell, usually ripping the sporangial wall, and escape as motile elements when functional flagella are synthesized. Flagellation and motility are separate phenomena, both of which lose intensity with age. Spores from old sporangia can regain motility when supplied with an exogenous carbon source, but, when provided only with water, phosphate buffer, or amino acids, flagellation takes place without motility. Deflagellation-reflagellation experiments indicated that functional flagella can be reformed only in presence of both amino acids and glucose which must be added within 180 min of deflagellation. Inoperative flagella were formed in the presence of inhibitors of nucleic acid synthesis, such as 6-azauracil, but inhibitors of protein synthesis, such as chloramphenicol, did not interfere with reflagellation. Flagellated spores remained so after germination.

Couch speculated that strains of *Actinoplanes* release their motile sporangiospores after a splitting of the sporangial envelope caused by the swelling of an intersporal substance (1). The presence of an intersporal substance was confirmed by the electron microscopic examination of sections of sporangia of *Actinoplanes* spp. at various stages of maturity (6, 7), but evidence supporting its role in the release of sporangiospores is lacking.

In the present study, the spore-releasing mechanism of *Actinoplanes* sp. 7-10 (7) was investigated, leading to the conclusion that motility and spore swelling are the main causes of dehiscence in this *Actinoplanes* sp.

MATERIALS AND METHODS

The methods used for the cultivation of *Actinoplanes* sp. 7-10 and its electron microscopic observation were as described previously (3). *Actinoplanes* sp. 7-10 cannot be identified with any of the presently described species of *Actinoplanes*. It forms longitudinal rows of sporangiospores, as do strains of *Ampullariella*, but the fact that it forms globose spores puts it squarely in the genus *Actinoplanes* (1).

Dehiscence of sporangia was observed in preparations made in welled slides or directly on plates. Sporangia were immersed in water or any appropriate solution, and enumeration of released sporangiospores was by hemocytometer counts of samples of fluid in which known numbers of sporangia had been bathed.

Spore suspensions. These were obtained by running a wire loop over the surface of flooded plate cultures to disrupt mature sporangia. Percentage of motile spores was determined by microscopic counts in a hemocytometer, and flagella were counted by electron microscopic examination. In both cases, counts were based upon populations of at least 200 spores.

Suspensions of sporangia. Such suspensions for micromanipulative dissection were obtained by gentle scraping of the surface of agar plates which had been flooded with water, followed by low-speed centrifugation (160 × *g* for 2 min) of the decanted suspension. The sediment was gently washed with 0.05 M phosphate buffer at (*p*H 7.0), which contained 1 μliter of polyoxyethylene sorbitan monooleate (Tween 80, Atlas Powder Co., Wilmington, Del.) per liter. The pellet obtained after centrifugation as above was rich in sporangia. These were dispersed in buffer, and microdrops of the suspensions were placed on cover slips coated with 2% agar which were then inverted over moist chambers. The sporangia were emptied with a microneedle attached to a Cailloux micromanipulator (C. H. Stoelting Co., Chicago, Ill.). When motile, the spores would swim in a small pool of buffer placed next to the sporangium undergoing surgery, and the percentage of motile spores was established by direct microscopic count.

Deflagellation. Spores were deflagellated by cooling to 4 C and vigorously agitating them for 12 min in a Waring Blendor equipped with a semimicro cup

modified with an ice jacket. The reflagellation medium consisted of 1 g of glucose and 1 g of Casamino Acids (vitamin-free; Difco) dissolved in 1 liter of 0.05 M phosphate buffer at pH 7.0. Protein determinations were made by a modification of the Folin-Ciocalteu method (8).

Spore volumes. These were estimated from electron microscopic measurements of freeze-dried preparations (10). Sporangia were suspended in neutralized ammonium acetate. A 1.6 molal ammonium acetate solution was neutralized by the addition of 1.07 g of sodium hydroxide per liter and was further diluted as needed. Spore suspensions were prepared by mechanically breaking the sporangia in this solution or in dilutions thereof.

Results and Discussion

When the dehiscence of sporangia from a 4- to 6-day-old agar plate culture of *Actinoplanes* sp. was observed in water, the total number of motile and nonmotile spores released approached a maximal value some 60 min after flooding (Fig. 1, A). The addition of a wetting agent, such as Tween 80, speeded up the dehiscence process, and maximal spore yields were observed within 45 min (Fig. 1, B). The beneficial action of the wetting agent came from the fact that the sporangia of *Actinoplanes* sp. fused into hydrophobic masses which were slowly penetrated by water. Furthermore, the sporangial envelope probably contained a hydrophobic layer, the properties of which were changed by Tween 80. Indeed, electron transparency of sporangial walls increased with Tween 80 treatment.

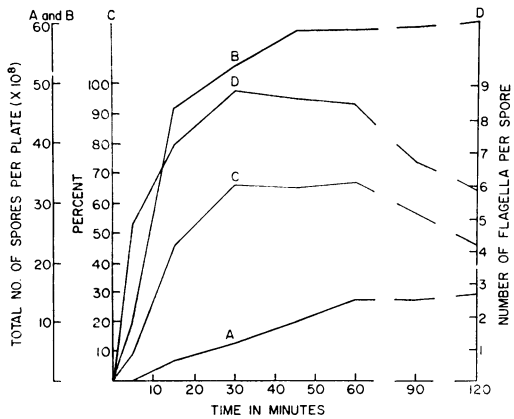

FIG. 1. *Dehiscence of sporangia of Actinoplanes 7–10 in water and 1 μliter Tween 80 per liter. Flagellation and motility of spores in water suspension. A, Total number of spores released after flooding a plate culture with water. B, Same after flooding with water to which Tween 80 was added. C, Percentage of motile spores in water suspensions of sporangiospores. D, Number of flagella per spore in above spore suspensions.*

When flooded sporangia were broken to make spore suspensions, the spores, which were at first mostly immobile and nonflagellated, became mostly motile and flagellated within 30 min (Fig. 1, C). As might be expected, motility lagged behind flagellation (Fig. 1, D).

When spore suspensions were prepared with 5% Formalin neutralized by calcium carbonate, only nonmotile, unflagellated spores were obtained. Hence, flagellation took place only after wetting. This point was confirmed by electron microscopic observation of spores removed by microdissection from sporangia before and after wetting. Again, the addition of Tween 80 accelerated flagellation.

The role of motility on dehiscence was assessed by poisoning the motility systems of spores within sporangia and subsequently noting the number of nonmotile spores released.

Spore suspensions prepared with buffered solutions of sodium *p*-chloromercuribenzoate, sodium iodoacetate, 2-iodoacetamide, sodium azide, and 2,4-dinitrophenol contained mainly unflagellated spores (Table 1). These inhibitors also immobilized fully motile spores. DeRobertis and Peluffo (2) also found that thiol inhibitors immobilized *Proteus vulgaris*, but that sodium azide was ineffective. Likewise, Kerridge (5) observed that sodium azide and 2,4-dinitro-

TABLE 1. *Effect of chemicals on flagellation, motility, and dehiscence of spores of Actinoplanes sp.*

Chemicals[a]	Spore suspensions		Approx per cent motile spores[b]	Dehiscence after 120 min	
	Per cent motile	Avg no. of flagella/spore		Approx per cent motile spores released[c]	Approx per cent nonmotile spores[d]
Na *p*-chloromercuribenzoate	0	0.1	0	0	10
Na iodoacetate	0	0.1	0	0	5
2-Iodoacetamide	0	0.1	0	0	5
Na azide	0	0	0	0	5
2,4-dinitrophenol	0	0	0	0	5
Na arsenate	59		50	50	50
Na cyanide	62		75	75	10
Na malonate	72		100	100	5
Na fluoride	76		100	100	5
Control-buffer[e]		8	100	100	

[a] All in control buffer at 10^{-2} M except chloromercuribenzoate at 10^{-3} M.

[b] Determined 60 min after addition of chemical; buffer control = 100%.

[c] Buffer control = 100%.

[d] Expressed as approximate percentage of motile spores released in buffer control.

[e] 0.05 M phosphate buffer (*p*H 6.5).

phenol did not reduce the motility of *Salmonella typhimurium*, although flagellation was prevented.

When sporangia were bathed in solutions of the above-mentioned inhibitors, only nonmotile spores were sparingly released. After 120 min, their number was only about 5% of that of motile spores in control preparations (Table 1).

It was also possible to obtain sporangia that released mainly nonmotile spores by bathing those from 8- to 10-day cultures in phosphate buffer containing either inhibitors of nucleic acid synthesis (6-azauracil or actinomycin D) or inhibitors of protein synthesis (chloramphenicol or streptomycin sulfate). The inhibitors of nucleic acid synthesis had no effect on younger sporangiospores, whereas the action of inhibitors of protein synthesis was already detectable (Table 2).

Since some nonmotile sporangiospores were released, under conditions where flagella were not formed (Table 1), or were nonfunctional when formed (Table 2), motility cannot be the only mechanism of dehiscence.

Spores increased in volume in the presence of water. Also, one notes a progressive increase in the numbers of spores released from sporangia, and in the motility and number of flagella per spore with a decrease in osmotic pressure (Table 3). The addition of Formalin to liquids used to prepare spore suspensions did not prevent the swelling of spores, but prevented flagellation and motility. Thus, the swelling of spores, which tripled in volume in presence of water, must be a factor in dehiscence. Microscopically, there was no indication that the intersporal substance swelled appreciably, as is seen in the formation of sporangial drops by some of the *Mucorales* (4). The sporangial envelope seemed to be readily permeable, and it did not collapse when soaked in solutions of high osmotic pressure.

The results in Table 2 suggested that flagellation and motility decreased in old spores because of the exhaustion of a pool of necessary metabolites. The addition of glucose to the liquid in which old sporangia were suspended permitted flagellation and enhanced motility (Table 4). Amino acids or phosphate buffer alone permitted flagellation without motility. Further, glucose had to be added to the spore-suspending medium within 180 min of the initial wetting if tangible benefit was to be obtained.

From quantitative spore population studies, it seems that, whereas some spores may have up to 40 flagella, a single, well-energized, flagellum may be sufficient for motility.

Deflagellation of spores was difficult, requiring treatment for 12 min in a Waring Blendor. Yet, after blending, direct counts of germinated spores indicated that they remained 99 to 100% viable. Reflagellation and motility did not take place in the absence of nutrients, and in this case Casamino Acids and glucose were both required.

TABLE 2. *Effect of inhibitors of nucleic acid and protein syntheses on the release of sporangiospores by Actinoplanes sp.*

Drug	Amt (μg/ml)	4–5 days[a]		8–10 days[a]	
		Motile spores (%)	Avg no. of flagella/spore	Motile spores (%)	Avg no. of flagella/spore
6-Azauracil	100	69	10	4	5
Actinomycin D	50	72	10	2	4
Chloramphenicol	100	26	7	1	2
Streptomycin sulfate	100	24	6	0	2
Control-buffer	—	67	9	34	6

[a] Age of cultures.

TABLE 3. *Effect of osmotic pressure on spore volume, flagellation, motility, and liberation of spores from sporangia*

Neutralized ammonium acetate (molality)	Spores 60 min after suspension			Dehiscence after 120 min[a]
	Vol (μ³)	Motile (%)	Avg no. of flagella/spore	
1.6	0.216	0	0.1	0.1
0.8	0.706	<1	0.2	5
0.4	0.706	7	0.4	10
0.2	0.706	38	7	50
Water control	0.984	80	8	100
Dry control[b]	0.294	—	—	—

[a] Expressed as percentage of water control.

[b] Measurements of spores made from freeze-dried disrupted sporangia which had not been suspended in a liquid.

TABLE 4. *Effect of nutrients on flagellation and motility of old (12 to 15 day) cultures of Actinoplanes sp.*

Suspending medium[a]	Spores 60 min after preparation of suspension		
	Motile (%)	Flagellated (%)	Avg no. of flagella/spore
Control-buffer	1	60	3
Glucose	67	66	3
Casamino Acids	1	59	4
Glucose + Casamino Acids	71	63	5

[a] Nutrients added to 0.05 M phosphate buffer (*p*H 7.0) at 1 g per liter.

Reflagellation was a slower process than initial flagellation, requiring 60 min for completion. Like flagellation, the amount of reflagellation depended on the total amount of time that the spores had been in contact with water prior to nutrient addition. The regeneration of motility and flagella in response to nutrient addition decreased after approximately 60 min and was usually zero after 180 min. A 100 μg/ml amount of 6-azauracil interfered significantly with reflagellation, but its main action appeared to be the production of nonfunctional flagella. Actinomycin D, chloramphenicol, and streptomycin, when used in the doses previously reported, had little effect on reflagellation and motility, but inhibited 40 to 50% of the overall protein synthesis of spores undergoing reflagellation. Thus, inhibitors of protein synthesis, such as chloramphenicol, did not interfere with reflagellation of the spores of the *Actinoplanes* sp. Similar results have been reported for other bacteria (9).

A small percentage of spores (<5%) germinated in glucose, but normal levels of germination required the addition of Casamino Acids as well as of glucose. Typically, the process began with the production of the germ tube and cessation of motility some 4 hr after initial wetting at 37 C. Flagella persisted after germination. Chloramphenicol and actinomycin D inhibited germination and lengthened the motile state, an effect that could be obtained also by lowering the temperature. In contrast, 6-azauracil stimulated germination, but growth was inhibited at the germ-tube stage.

This study leads to the general conclusion that the sporangial envelope of *Actinoplanes* sp. is water repellent, but that, once it is wetted, it is fully permeable to water and solutions of various osmotic pressures. Swelling of the sporangiospores in the presence of water is probably the main factor responsible for the splitting of the sporangial wall. Wetting of the spores is usually followed by flagellation and motility. The motion of the spores adds to the effect of the swelling and speeds dehiscence. Against this theory of the mechanism of dehiscence is the not infrequent observation that sporangia bathed in water may harbor actively motile spores that are not released. This probably happens when the sporangial sac is roomy enough to accommodate the swelled spores.

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