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Sporulation of *Streptomyces roseosporus* in submerged culture

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SUMMARY

A soil isolate of *Streptomyces roseosporus* was found to produce spores in stirred submerged culture. Both biological mass and respiratory activity increased during the sporulation process. Contrary to other reports, the differentiation process was not purposefully initiated by critical manipulation of either nutritional or environmental conditions.

INTRODUCTION

The life cycle of a typical *Streptomyces* species on solid media involves spore germination, and the development of vegetative mycelia followed by aerial hyphae that differentiate into chains of spores. Various substances have been reported to exert some control on the *Streptomyces* life cycle. Efremenkova et al. [2] have described the A-factor as a regulator of differentiation. Pamamycin has been reported to stimulate the formation of aerial hyphae [9]. The same type of activity has been described in a series of fungal culture broths [7]. It appeared that biotin was implicated in the activity found in the fungal cultures. In two *Streptomyces* mutants, Liu [8] reported that the formation of aerial hyphae was pH-dependent. Carbazomycinal and 6-methoxy-carbazomycinal have been reported to inhibit the formation of aerial hyphae in a species of the closely related genus *Streptoverticillium* [6]. The formation of spores under submerged conditions in members of the genus *Streptomyces* is an uncommon occurrence. There are very few reports on that phenomenon. Kendrick and Ensign [5] have described the induction of submerged sporulation in *Streptomyces griseus* by either nitrogen or phosphorus limitation.

During our studies on the production of the A21978C complex of antibiotic compounds by strains of *Streptomyces roseosporus* [4], we observed that the original soil isolate produced spores in submerged culture. That sporulation process occurred very rapidly without critical nutritional or environmental conditions being applied. This report describes the conditions under which *S. roseosporus* produces submerged spores and establishes that biological mass continues to increase during the differentiation process.

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MATERIALS AND METHODS

The culture used in this study was a strain of *Streptomyces roseosporus* isolated from soil. That culture was found to produce the A21978C lipopeptide antibiotic complex. To ensure homogeneity the culture was plated on a medium containing trypticase soy broth (3.0% w/v), potato dextrin (2.5% w/v), agar (2.0% w/v) and deionized water. The medium was sterilized at 121°C for 20 min and then 20 ml were added to each Petri dish. When the medium reached ambient temperature, the culture was streaked across its surface and the plate was incubated at 30°C for 150 h. Two slant cultures of the same medium were inoculated from a single isolated colony and incubated for 240 h at 30°C.

A 2 l Erlenmeyer flask containing 300 ml medium was used as the primary agitated inoculum development reactor. The medium contained trypticase soy broth (3.0% w/v), potato dextrin (2.5% w/v) and deionized water. The flask was sterilized at 121°C for 30 min, cooled to ambient temperature and inoculated with the growth from one-half of a slant culture suspended in 2.5 ml of the flask medium. The flasks were incubated at 32°C for 120 h on a rotary shaker at 250 rpm (2″ throw). The contents of two flasks were used to inoculate the secondary agitated inoculum development reactor. That reactor was constructed of stainless steel and had an operating and total volume of 1900 l and 2500 l, respectively. The medium within the reactor was stirred (1 hp/100 gal), aerated (1 vvm) and maintained at 30°C. The medium used in that reactor contained glucose (2.0% w/v), silicone antifoam 471 (0.04% w/v), calcium carbonate (0.2% w/v), soybean flour (2.0% w/v), yeast extract (0.1% w/v), potassium chloride (0.02% w/v), magnesium sulfate heptahydrate (0.02% w/v), ferrous sulfate heptahydrate (0.0004% w/v) and tap water. The medium was sterilized at 121°C for 45 min.

After 21 h of incubation, 12 l of the secondary inoculum development stage were added to 108 l of medium contained in a stainless steel 150 l reactor. The medium used in the final culture stage contained methyl oleate (3.0% w/v), silicone antifoam 471 (0.22% w/v), potassium chloride (0.1% w/v), yeast extract (0.1% w/v), diammonium phosphate (0.87% w/v), magnesium sulfate heptahydrate (0.005% w/v), zinc sulfate heptahydrate (0.0013% w/v), cupric sulfate pentahydrate (0.0014% w/v), cobalt chloride hexahydrate (0.0005% w/v) and tap water. The pH of the medium was adjusted to 7.0 with sodium hydroxide and sterilized at 121°C for 45 min. The operating conditions for the final culture reactor are presented in Table 1. The pH was controlled only with 29% ammonium hydroxide.

Table 1

Conditions for operating the final culture reactor

Character	Hour	Value
Temperature	0	34°C
	10	30°C
pH set-point control	0	6.5
Agitation	0	250 rpm
	10	350 rpm
Aeration	0	2.1 cfm
Glycerol	5	30 ml/h
Volume	0	120 l

The respiratory activity of the culture was estimated by analyzing the exit gas stream from the reactor with a Perkin Elmer mass spectrometer. Oxygen demand and carbon dioxide evolution were calculated by comparison of standard gas mixtures with those same gasses in the atmosphere and exit stream. The respiratory quotient was calculated from the oxygen demand and carbon dioxide evolution. To insure that adequate oxygen was always present, dissolved oxygen was monitored by a dissolved O_2 probe (Leeds and Northrup).

Biological mass was estimated by both dry cell weight and colony forming units. The dry weight estimate involved centrifuging 10 ml of culture fluid at 1600 × *g* for 10 min. The supernatant was discarded and the pellet suspended in 10 ml acetone to remove any contaminating lipid. The suspension was centrifuged as before and the supernatant discarded. In order to remove any inorganic precipitates, the pellet was suspended in 10 ml of 2 N HCl and subjected to centrifugation and decantation. Following two washings with 0.85% NaCl by cen-

trifugation and decantation, the solids were transferred in distilled water to a tared weigh boat and dried to a constant weight at 70°C. Colony forming units were estimated by diluting the culture in sterile 0.85% (w/v) NaCl and plating the diluted sample on the previously described solid medium. The plates were incubated at 30°C for 72 h and the growth centers counted.

Inorganic phosphate and ammonia nitrogen were automatically determined by the 'Industrial Method No. 93-70W' (Technicon Industrial Systems, Tarrytown, NY). Total lipid was estimated by acidifying a known amount of culture fluid and extracting that mixture with isopropanol/heptane (1:1, v/v). The heptane layer was transferred to a tared weigh boat and allowed to dry under ambient conditions. The increase in the weight of the boat was mathematically related to the amount of total lipid present in the culture fluid.

Culture fluids were prepared for microscopic examination by harvesting an aliquot of whole broth from the reactor vessel at appropriate periods and washing several times by centrifugation to remove extraneous material. For observation under light microscopy, wet mounts were made and photomicrographs were taken directly under oil immersion using phase contrast at 1600 magnifications. A Reichert Zetopan research microscope equipped with Kodak Technical Panchromatic film rated as ASA 64 was used to document morphology. Scanning electron micrographs were made by filtering whole broth onto a Nucleopore® filter which was then critical point dried. The sample was rotary shadowed with platinum-gold and observed using a scanning electron microscope.

Submerged spores were examined for diaminopimelic acid using chromatographic methods described by Becker et al. [1].

RESULTS AND DISCUSSION

The data from the analysis of various parameters during a 36 h culture of *S. roseosporus* are presented in Tables 2 and 3. Since the pH was above the set-point for the pH control mechanism during the initial 12 h of culture, no additional ammonium ions were added to the medium. During that same period the residual ammonia in the broth decreased. After 12 h, the pH control system added ammonium hydroxide to the culture. Thus, after 12 h, residual ammonia could not be accurately related to cellular activity. Residual phosphate and lipid decreased during the entire 36 h process.

The respiratory acivity of the culture increased markedly with time. Oxygen demand increased almost four-fold and carbon dioxide evolution doubled during the 36 h of cultivation. That adequate oxygen was available to *S. roseosporus* was indicated by the continued increase in oxygen demand and the very high value for dissolved oxygen. Either oxygen demand or oxygen demand and growth was responsible for the continued decrease in dissolved oxygen during the period under investigation. The unusual respiratory quotient (RQ) suggested that the culture was using mixed carbon sources during the early hours of cultivation. Most probably, methyl oleate and organic nitrogenous compounds were being used during that period. The extremely low RQ after 12 h indicated that the

Table 2

Changes in biological mass and chemical characteristics of sporulating cultures of *S. roseosporus*

Time (h)	Dry weight (mg/ml)	Growth centers (No./ml)	pH	Phosphate (mg/ml)	Ammonia (mg/ml)	Total lipid (mg/ml)
0	1.2	1.1×10^7	6.8	2.29	1.23	26.6
12	2.7	1.2×10^8	6.5	2.23	0.87	24.2
24	5.1	1.7×10^8	6.4	2.06	0.75	23.3
36	8.5	5.4×10^9	6.5	1.98	0.76	18.1

Table 3

Respiratory activity during sporulation of *S. roseosporus*

Time (h)	Dissolved O_2 (%)	Oxygen uptake (mm/l/h)	CO_2 evolution (mm/l/h)	Respiratory quotient
0	95	0.10	0.08	0.80
6	77	0.20	0.16	0.80
12	77	0.13	0.09	0.69
18	74	0.22	0.12	0.55
24	70	0.28	0.14	0.50
30	69	0.32	0.15	0.47
36	67	0.38	0.18	0.47

culture was utilizing the methyl oleate and conserving large amounts of carbon. Such carbon conservation and increased respiratory activity indicates that the culture was continuing to grow. The two estimates of biological mass confirmed this. Mass, as indicated by dry cell weight, increased continuously throughout the process. A significant increase in growth centers was also observed during the

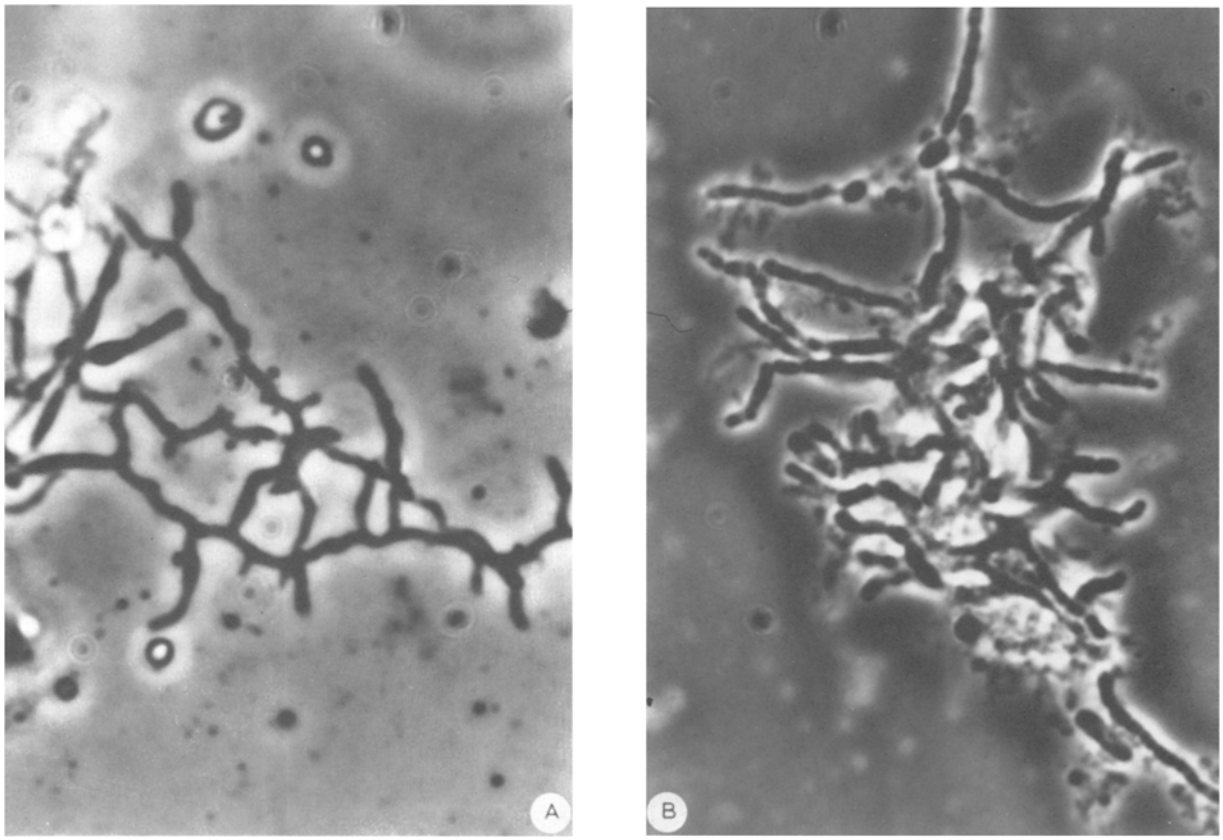


Fig. 1. Light microscopic observations of submerged sporulation of *S. roseosporus*. A, B, C and D represent the culture at 0, 12, 25 and 36 h, respectively.

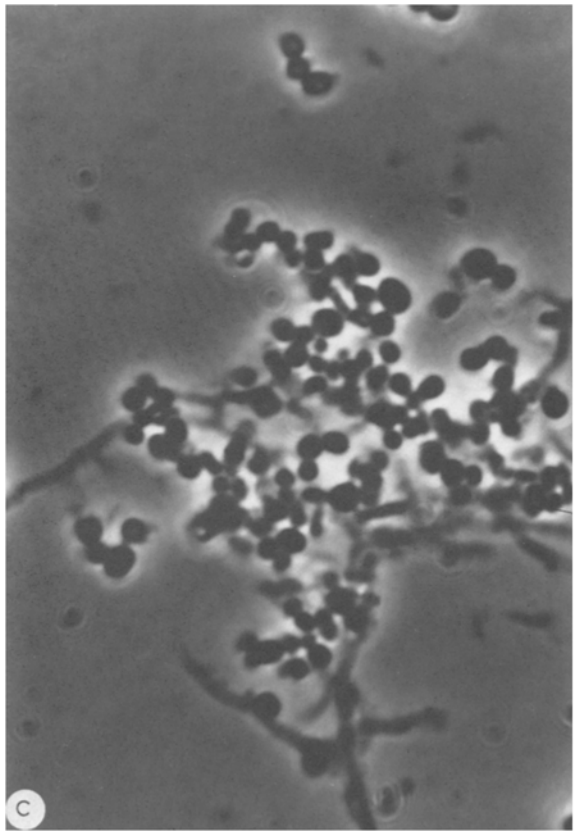
period of cultivation. The change in the latter parameter could have been related to a true increase in vegetative cell number or it could also represent hyphal fragmentation and sporulation.

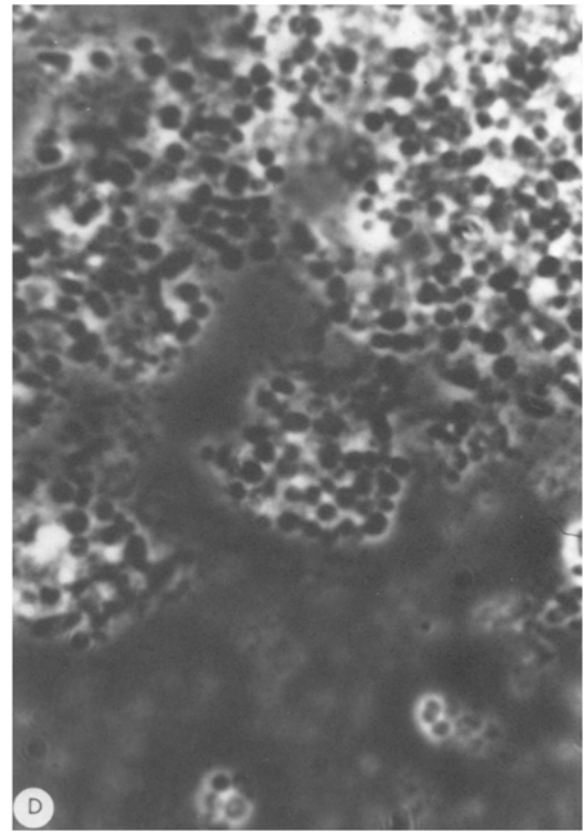
When the same samples were examined microscopically, it was observed that differentiation had clearly taken place. Fig. 1 (A–D) shows the development of submerged spores by *S. roseosporus* during 36 h of cultivation in a stirred reactor. Branched vegetative filaments were the dominant form during the early hours of cultivation. That morphology is characteristic of *Streptomyces* grown under submerged conditions. As the time of incubation increased, greater spore populations were noted. These spores were viable when plated. The final sample from the stirred culture appeared to only contain spores. Electron micrographs (Fig. 2, A–D) confirmed the light microscopic observations. The formation of submerged spores did not occur in either the primary of secondary media, but only in the fermentation medium containing methyl oleate.

Since some genera of the *Actinomycetales* have been reported to contain both the L- and meso-isomers of 2,6-diaminopimelic acid [10], the submerged spores of *S. roseosporus* were examined for those compounds. They were found to contain only the LL-isomers, which is diagnostic for *Streptomyces*.

When grown on solid agar media, typical chains of spores borne on aerial hyphae were observed. An electron micrograph of these aerial spores is shown in Fig. 3.

Ensign [3] stated that spore formation by Streptomycetes in submerged culture was a controversial point, and at that time (1978) he had never observed sporulation in submerged culture. A later





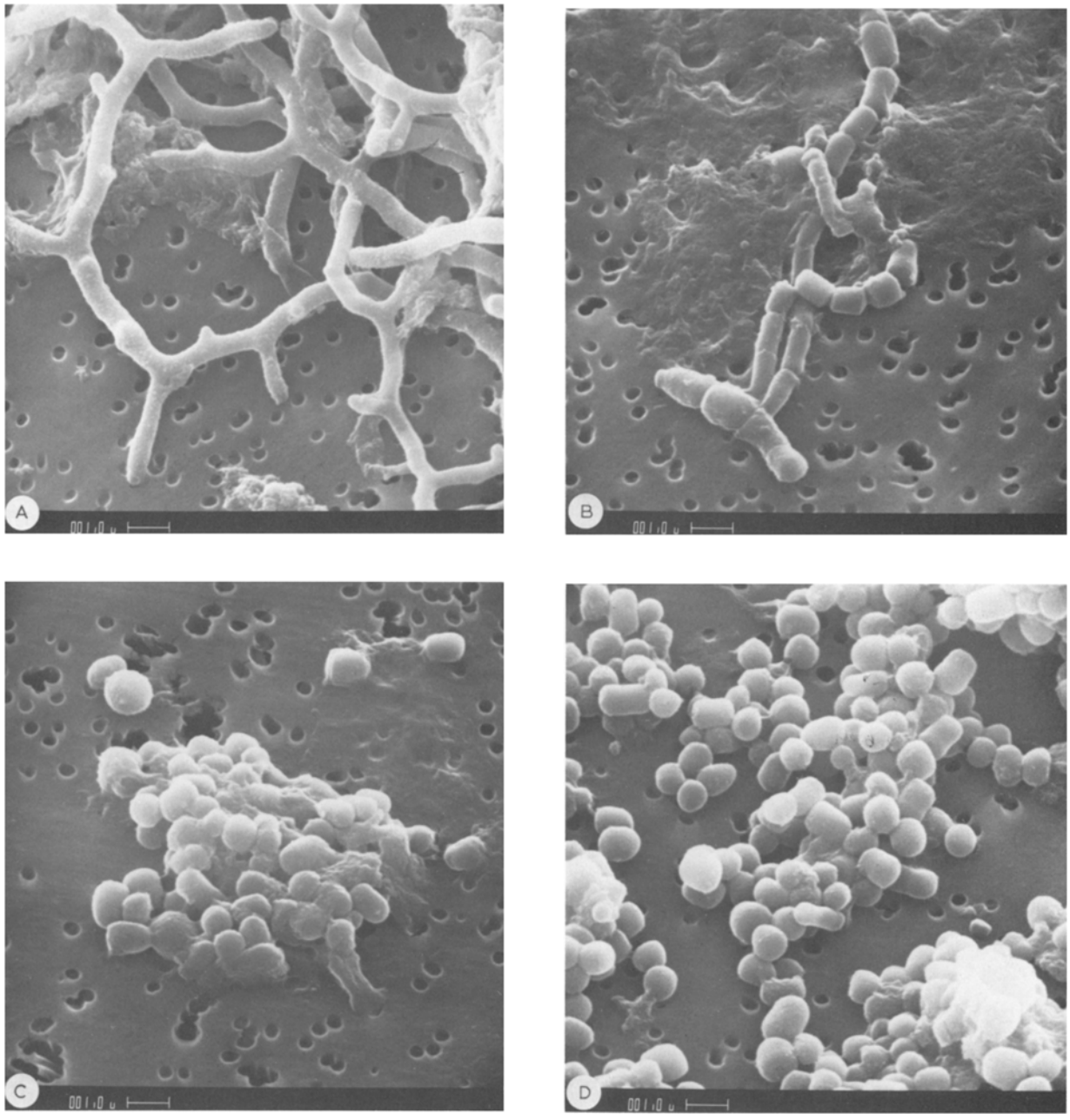


Fig. 2. Scanning electron microscopic observations of the submerged sporulation process of *S. roseosporus*. A, B, C and D represent the culture at 0, 12, 25 and 36 h, respectively,

report involving the same author [5] demonstrated that either nitrogen or phosphorus starvation could initiate submerged spore formation in a strain of *Streptomyces griseus*. In contrast to the work with *S. griseus* where submerged spore formation did not begin until 36 h, the production of submerged spores by *S. roseosporus* is unique in that sporulation was initiated earlier and the process was not dependent on deliberate nutrient deprivation.

Since others [2] have described substances that

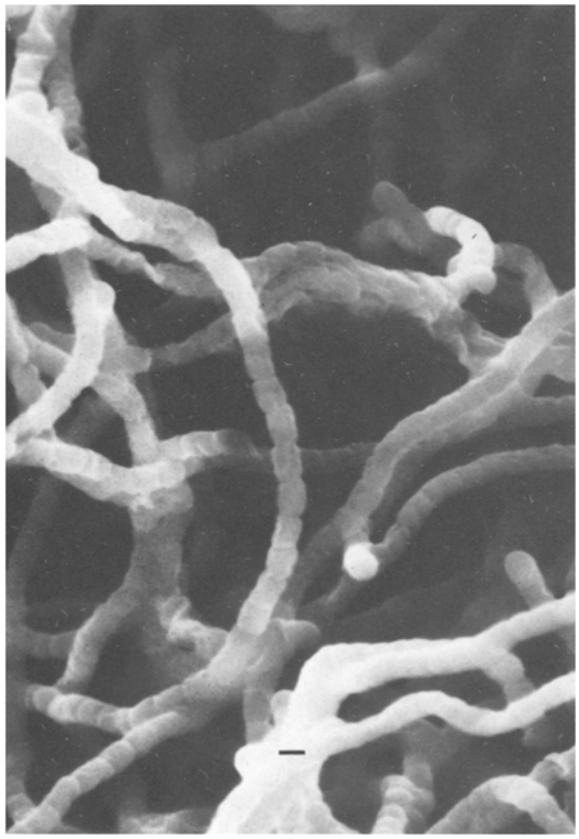


Fig. 3. Scanning electron micrograph of aerial spores of *S. roseosporus* grown on solid media.

regulate cell differentiation in Streptomycetes, it is possible that a similar regulator(s) might initiate spore formation in *S. roseosporus*. The sporulation process in *S. roseosporus* will be characterized further in a future investigation.

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