ELECTRON MICROSCOPY OF STREPTOMYCES SPORE MORPHOLOGY AND ITS ROLE IN SPECIES DIFFERENTIATION

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The widespread search for new antibiotic-producing Streptomyces unleashed during the last two decades also brought with it the problems of their classification. In the early attempts at species differentiation excessive use was made of highly variable cultural and physiological properties as taxonomic criteria and too little attention was paid the more stable morphological characteristics. However, in some of the more recently derived Streptomyces classification systems there has been increasing evidence that certain morphological properties are finally and properly being accorded major status as criteria for species differentiation.

Pridham, Hesseltine, and Benedict (1958) assigned prime importance to the morphology of Streptomyces sporophores and grouped species into primary morphological sections on this basis. In the system of Ettlinger, Corbaz, and Hütter (1958) the type of sporophore (spore chain) was also one of the characteristics given major emphasis but the morphology of the spores themselves, as observed by electron microscopy, was given equal weight. In recent years several other investigators (Flaig et al., 1952; Küster, 1953; Flaig and Kutzner, 1954; Flaig, Küster, and Beutelspacher, 1955; Baldacci and Grein, 1955; Baldacci, Gilardi, and Amici, 1956; Kutzner, 1956; and Preobrajenskaya et al., 1959) using the electron microscope have studied the details of Streptomyces spore structure and have shown that an apparently distinctive spore surface configuration exists in many of the species. Provided with this encouraging prospect, it seemed highly desirable to expand these studies to include mass collections of strains of individual *Streptomyces* species, for it is only by demonstration of constancy from strain to strain within a species that the true value of any criterion for species differentiation can be firmly established. Having available substantial numbers of strains of many species of this genus, which in the past were used to demonstrate that H_2S production merits major status in Streptomyces systematics (Tresner and Danga, 1958), it was logical to make use of this assemblage of organisms again in the further evaluation of spore ornamentation as a criterion.

MATERIALS AND METHODS

The RCA EMU 3C electron microscope was employed in the present study for observing Streptomyces spore micromorphology. Preparations for examination were made by a simple spore print technique in which Formvar-covered copper grids (200 mesh) (Shawinigan Resin Corporation, Springfield, Massachusetts) were gently pressed to the sporulating surface of the organisms. Spore chains adhering to the surface of the grids could be viewed in the microscope without the necessity of fixing or shadowing the material. Because of the simplicity of this method, as many as 10 cultures per hour could be studied. Most observations were made at about 8,000 enlargements which permitted easy visibility of the nature of the spore surfaces, and which was generally satisfactory for making electron micrographs.

Cultures for study were grown in petri dishes for 14 days at 28 C. A yeast-malt agar medium [0.4 per cent yeast extract (Difco), 1.0 per cent malt extract (Difco), 0.4 per cent glucose, 2.0 per cent agar, and 1.0 liter distilled H_2O; adjust to pH 7.0] that usually induced good sporulation was used routinely; however, in some instances when sporulation was scanty or absent on this medium, a Waksman's starch agar (Waksman, 1957) or a tomato paste oatmeal agar (Pridham et al., 1957) was employed. Mature cultures were preserved with formalin (Tresner and Backus, 1957) and stored at 4 C until needed.

RESULTS

During the course of our study approximately 600 Streptomyces strains were examined, among which were present some 118 described species

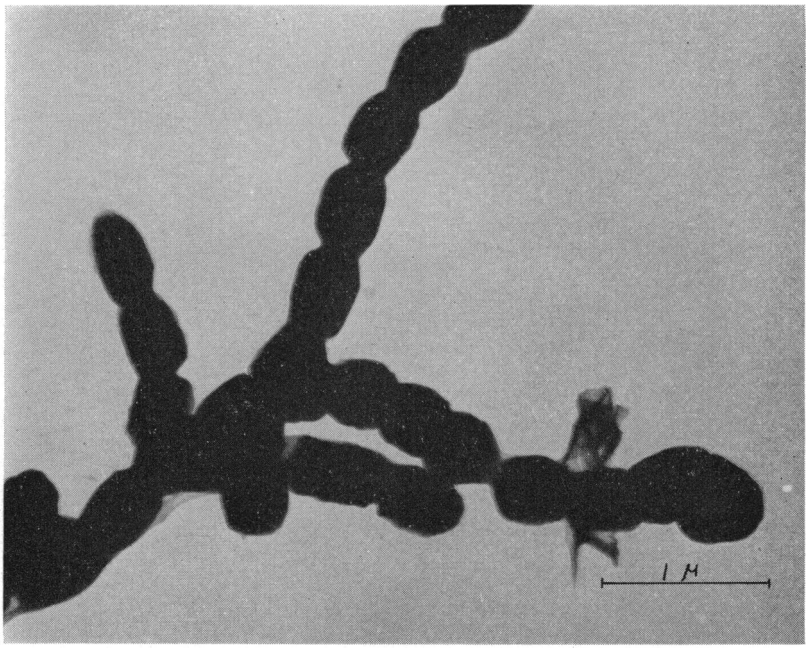

Figure 1. Smooth spores of *Streptomyces diastaticus* (ATCC 3315)

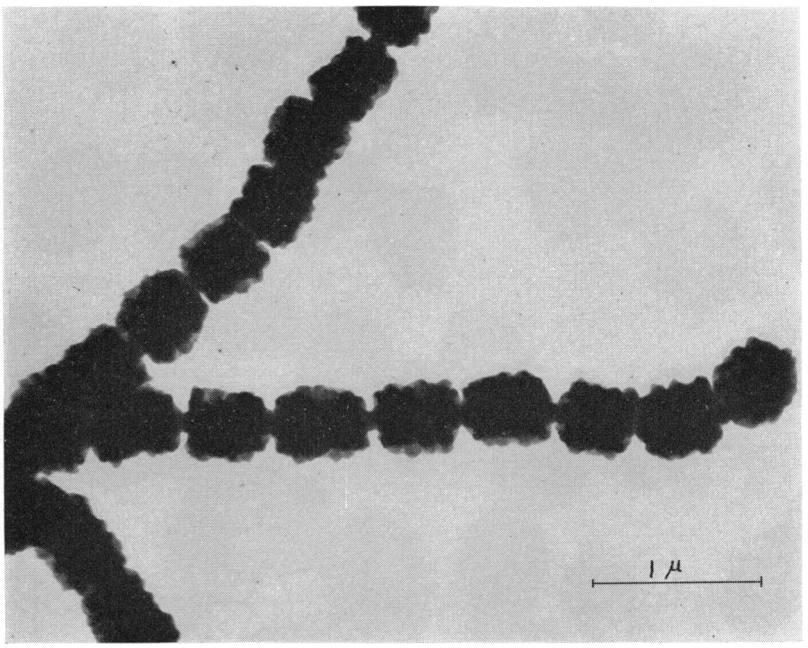

Figure 2. Obtuse protuberances on the wall surface of spores of *Streptomyces olivaceus* (ATCC 12019)

or varieties. About 350 of the strains were assignable to a group of 25 species, in which each species was represented by a block of 5 to 40 (average 14) carefully selected strains that generally included one or more reference cultures obtained from various culture repositories.

It was observed that the spores of all the cultures studied could be classified according to

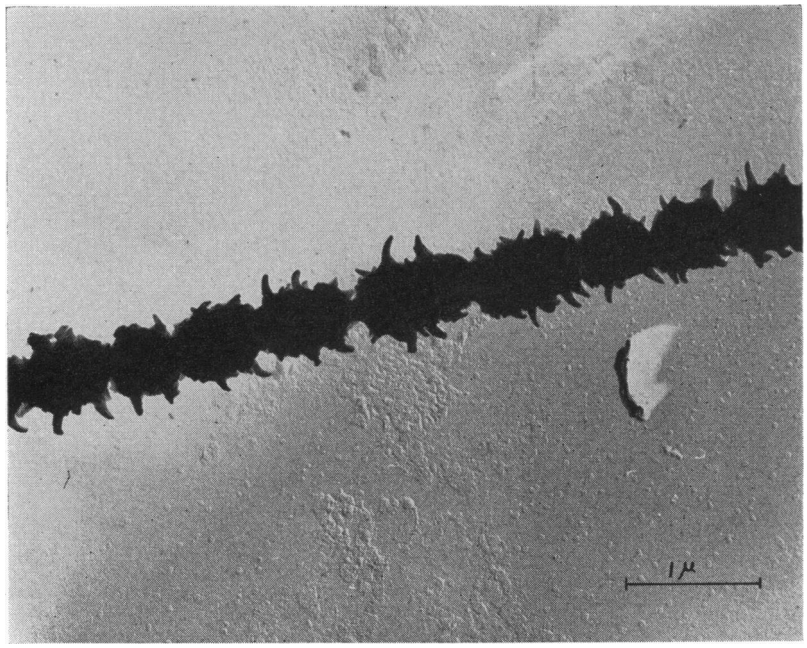

Figure 3. Spiny spores of *Streptomyces purpurascens* (NRRL B-1480)

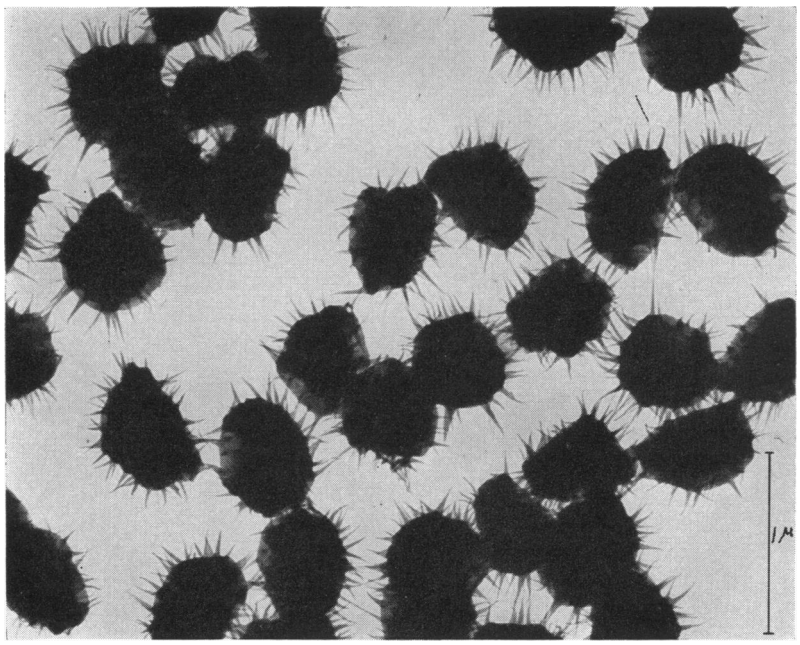

Figure 4. Spiny spores of *Streptomyces diastatochromogenes* (NRRL B-1698)

one or another of four morphological types, smooth, warty, spiny or hairy; this substantiates the findings of Kutzner (1956). By definition, we considered spores as smooth whenever there was no surface ornamentation present (fig. 1). Also included here were spores that sometimes showed considerable lumpiness or irregularities within the confines of the cell wall, but in which the

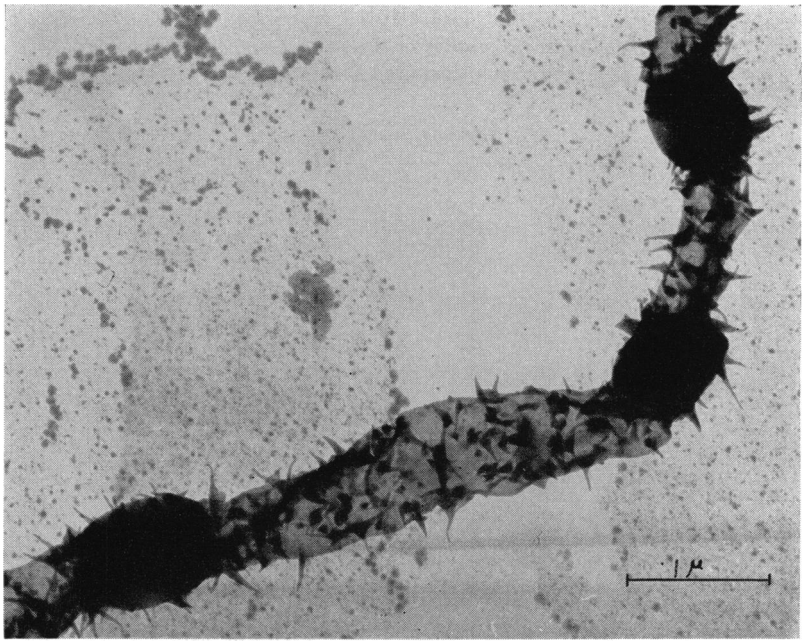

Figure 5. Spiny spores of *Streptomyces erythraeus* (NRRL B-2359)

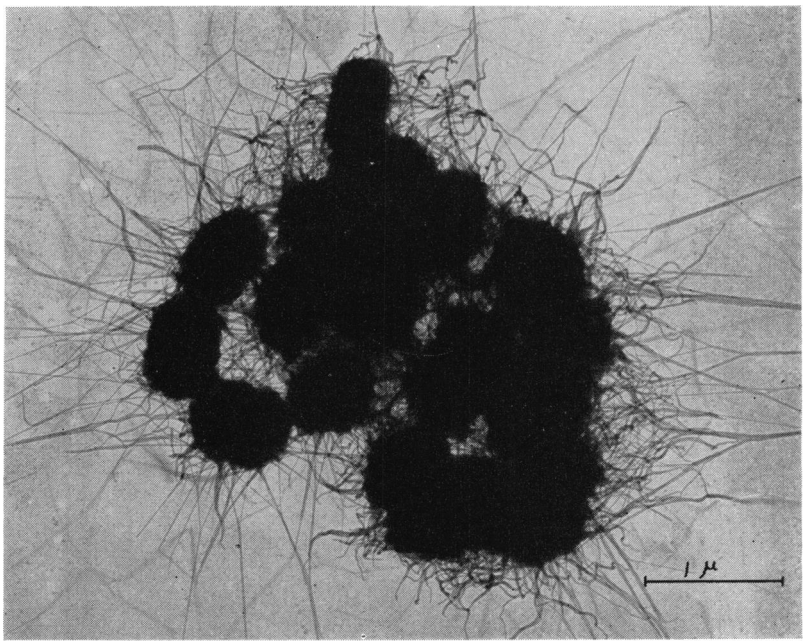

Figure 6. Long hairy outgrowths from spores of *Streptomyces albogriseolus* (NRRL B-1305)

surface contour was unmodified. Warty spores, on the other hand, had distinct, obtuse protuberances that were uniformly present on the wall surfaces (figure 2). Spiny spores, distinguished by acute, rigid appendages varying in length and thickness (figures 3, 4, and 5), were differentiated from hairy spores; the latter possessed thin, flexible, filiform outgrowths that sometimes

TABLE 1

Electron microscope studies of Streptomyces spore morphology

A. Smooth-spored Streptomyces

Organism	Total No. Strains Studied	Culture Collection Source and No.	Spore Color Group*
S. achromogenes	1	ATCC 12767	G-B
S. acidomyceticus	1	ATCC 11611	TAN
S. albidoflavus	4	NRRL B-1271	YCB
		CBS	YCB
		CBS (Duché)	YCB
		CBS (de Vries)	YCB
S. albireticuli	2	IFO 3400	YCB
		NRRL B-1670	YCB
S. alboniger	1	ATCC 12461	W
S. albosporeus	1	NRRL B-1239	YCB
S. albus	7	ATCC 3361	YCB
		ATCC 3351	YCB
		NRRL B-683	YCB
		ATCC 3004	W
		ATCC 3006	YCB
		ATCC 3005	W
		ATCC 618	W
		NRRL B-2401	TAN
S. ambofaciens	1	Rhone-Poulenc S.h.M-181-110	G-B
S. anulatus	1	NRRL B-1337	YCB
S. antibioticus	11	ATCC 8663	G-B
		ATCC 10382	G-B
		ATCC 11891	G-B
S. argenteolus	1	ATCC 11009	G-B
S. aureofaciens	40	ATCC 10762	G-B
		ATCC 12416a	G-B
		NRRL B-1286	G-B
		NRRL B-1287	G-B
		NRRL B-1288	G-B
S. aureus	2	ATCC 3309	G-B
		NRRL B-1265	G-B
S. bikiniensis	18	NRRL B-1049	G-B
		ATCC 11062	G-B
S. cacaoi	1	NRRL B-1220	YCB
S. caeruleus	1	NRRL B-1623	G-B
S. candidus	1	NRRL B-1571	YCB
S. canescus	2	NRRL B-2419	YCB
S. catenulae	1	ATCC 12476	G-B
S. cellulosae	1	NRRL B-1222	YCB
S. chrysomallus	1	ATCC 11523	YCB
S. cinnamonensis	1	ATCC 12308	TAN
S. citreus	1	ATCC 10974	YCB
S. coelicolor	1	CBS (Beijerinck)	YCB
S. crystallinus	26	Loewe	TAN
S. diastaticus	2	CBS (Waksman and Curtis)	YCB
		ATCC 3315	YCB
S. diastatochromogenes	1	Wis. M-248	G-B
S. erythraeus	2	ATCC 11912	YCB
		CBS (Duché)	YCB
S. eurocidicus	1	NRRL B-1676	YCB
S. exfoliatus	1	ATCC 12627	TAN

TABLE 1—(*Continued*)

Organism	Total No. Strains Studied	Culture Collection Source and No.	Spore Color Group*
S. felleus	18	NRRL B-2251	G-B
S. fimicarius	1	CBS (Duché)	G-B
S. flavovirens	1	NRRL B-1329	G-B
S. flavus	2	CBS (Waksman and Curtis)	G-B
		ATCC 3369	G-B
S. fradiae	5	Waksman (Neomycin)	TAN
		ATCC 11903	TAN
		ATCC 10745	TAN
S. fulvissimus	12	NRRL B-1453	TAN
S. gelaticus	1	NRRL B-1252	G-B
S. gougeroti	2	NRRL B-1344	YCB
		ATCC 10975	YCB
S. griseocarneus	2	ATCC 12628	TAN
		Hosoya H-365	TAN
S. griseolus	2	NRRL B-1062	G-B
		IFO 3403	G-B
S. griseoluteus	1	ATCC 12768	G-B
S. griseoroseus	1	ATCC 12125	YCB
S. griseoruber	1	NRRL B-1818	G-B
S. griseus	21	Waksman	YCB
		ATCC 10137	YCB
		ATCC 3326	YCB
		Waksman 3527	YCB
		ATCC 10971	YCB
		ATCC 11746	YCB
		NRRL B-150	YCB
S. griseus f. *farinosus*	1	NRRL B-1354	YCB
S. griseus var. *purpureus*	10	ATCC 3312	YCB
		NRRL B-2285	YCB
		NRRL B-2423	YCB
		NRRL B-1381	YCB
S. griseus var. *spiralis*	1	ATCC 13733	YCB
S. halstedii	3	CBS (Duché)	G-B
		NRRL B-1238	G-B
S. hiroshimensis	1	NRRL B-1823	TAN
S. hominis	1	ATCC 3008	YCB
S. hygroscopicus	27	CBS	G-B
		NRRL B-1346	G-B
		NRRL B-1503	G-B
		ATCC 10976	G-B
		IFO 3401	G-B
S. intermedius	1	NRRL B-1327	YCB
S. kentuckensis	1	NRRL B-1831	TAN
S. kitasatoensis	1	Hata	YCB
S. lavendulae	23	Waksman	TAN
		Waksman strain 8	TAN
		ATCC 8664	TAN
		ATCC 11924	TAN
S. lipmanii	2	ATCC 3331	YCB
		CBS (Waksman and Curtis)	YCB
S. microflavus	1	CBS (Krainsky)	YCB
S. mirabilis	1	Ankerman Co., Germany	G-B
S. naganishii	1	NRRL B-1816	G-B

TABLE 1—(*Continued*)

Organism	Total No. Strains Studied	Culture Collection Source and No.	Spore Color Group*
S. narbonensis	2	CBS (Corbaz)	G-B
		NRRL B-1680	G-B
S. netropsis	12	NRRL B-2268	TAN
S. nitrificans	1	NRRL B-1664	W
S. nitrosporeus	2	Okami O-20	G-B
		IFO 3362	G-B
S. odorifer	1	NRRL B-1328	YCB
S. olivaceus	2	ATCC 3335	G-B
		ATCC 11626	G-B
S. olivochromogenes	1	NRRL B-1341	G-B
S. parvulus	1	ATCC 12434	G-B
S. parvus	2	NRRL B-1455	YCB
		ATCC 12433	YCB
S. phaeochromogenes	4	NRRL B-1248	TAN
		IFO 3105	G-B
		IFO 3149	G-B
S. phaeofaciens	1	NRRL B-1516	G-B
S. pluricolorescens	1	Okami 91-T1-1	YCB
S. praecox	1	ATCC 3374	YCB
S. purpureochromogenes	6	ATCC 3313	YCB
		ATCC 3343	YCB
		CBS (Waksman and Curtis)	YCB
S. purpeofuscus	1	NRRL B-1817	G-B
S. reticuli	1	ATCC 3343	YCB
S. rimosus	13	ATCC 10970	W
S. rochei	9	NRRL B-1559	TAN
		ATCC 10739	TAN
S. roseochromogenes	5	CBS	TAN
		ATCC 3347	YCB
		Squibb SC 1624	YCB
		IFO 3363	TAN
		IFO 3411	TAN
S. roseoflavus	2	Kuroya #320	TAN
S. ruber	1	ATCC 3348	W
S. rubrireticuli	7	NRRL B-1484	TAN
S. rubrocyanodiastaticus var. *piger*		NRRL B-1775	G-B
S. rutgersensis	1	NRRL B-1256	YCB
S. salmonicida	2	NRRL B-1472	YCB
S. sulphureus	1	NRRL B-1627	YCB
S. tanashiensis	11	Hata #144	G-B
S. venezuelae	6	ATCC 10595	TAN
		ATCC 10712	G-B
		Waksman 3627	G-B
		Waksman 3629	TAN
S. verticillatus	4	ATCC 13017	YCB
		ATCC 13495	YCB
		ATCC 13538	YCB
		ATCC 13539	YCB
S. violaceoniger	3	NRRL B-205	G-B
		NRRL B-1476	G-B
		NRRL B-1477	G-B

TABLE 1—(*Continued*)

Organism	Total No. Strains Studied	Culture Collection Source and No.	Spore Color Group*
S. violaceoruber	5	Cochrane strain A25S	G-B
		Sermonti strain P (1)	G-B
		ATCC 3355	G-B
		ATCC 10147	G-B
S. virginiae	3	NRRL B-1446	TAN
		ATCC 12630	TAN
		IFO 3392	TAN
S. viridis	1	ATCC 3372	G-B
S. willmorei	2	ATCC 3332	YCB
		NRRL B-1332	YCB
B. Spiny-spored Streptomyces			
S. alboflavus	1	ATCC 12626	G-B
S. albus	1	NRRL B-2490	G-B
S. arabicus	1	NRRL B-1733	G-B
S. chartreusis	12	NRRL B-2287	BL-G
S. diastatochromogenes	1	NRRL B-1698	G-B
S. erythraeus	5	NRRL B-2338	TAN
		NRRL B-2359	TAN
		NRRL B-2360	TAN
		NRRL B-2361	TAN
S. filipinensis	1	CBS	G-B
S. gilvosporeus	1	ATCC 13326	G-B
S. griseoflavus	1	CBS (Ciferri)	G-B
S. noursei	1	ATCC 11455	G-B
S. pseudogriseolus	1	ATCC 12770	G-B
S. purpurascens	18	NRRL B-1454	TAN
		NRRL B-1480	TAN
S. viridochromogenes	9	NRRL B-1511	BL-G
		ATCC 3356	BL-G
Streptomyces sp.	14	Gottlieb (Levomycin)	G-B
Streptomyces sp.	11	Kuroya F-300 (Phagostatin)	G-B
Streptomyces sp.	1	NCIB 8697	G-B
C. Hairy-spored Streptomyces			
S. albogriseolus	12	NRRL B-1305	G-B
S. calvus	1	ATCC 13382	G-B
S. flaveolus	1	ATCC 3319	G-B
Streptomyces sp.	1	Robbins A419 (Chrysomycin)	G-B
D. Warty-spored Streptomyces			
S. diastaticus	1	NRRL B-2650	G-B
S. griseoplanus	4	Lederle AA-223	G-B
S. olivaceus	1	ATCC 12019	G-B

* G-B = spores in shades of gray, gray-brown or brown; YCB = spores in shades of yellow, cream or buff; TAN = spores in shades of pinkish-tan to pinkish-cinnamon; BL-G = spores in shades of blue to bluish-green; and W = spores white or cultures with pure white aerial mycelium and not sufficient sporulation to impart a color.

attained lengths several times the greatest dimension of the spores (figure 6).

From an examination of table 1 it will be noted that smooth spores were by far the most commonplace and were characteristic of 80 per cent of the species studied. In streptomycetes having spore color in yellow to cream to buff or white shades, only smooth spores were found. Although smooth spores were the rule in the organisms having pinkish-cinnamon to pinkish-tan sporulation, *Streptomyces erythraeus* (Waksman) Waksman and Henrici, *Streptomyces purpurascens* Lindenbein, and two undetermined species belonging to this spore color group were outstandingly different with their spiny spores. About one-third of the gray to brownish-spored forms possessed distinctive spines, hairs or warts, although the latter two types were relatively rare. All of the cultures having blue to blue-green spore masses consistently produced spiny spores.

In keeping with the findings of Ettlinger et al. (1958) as well as other investigators, we observed that organisms having ornamented spores, produced them in the form of spiralled chains (figure 7) or less commonly in loops or coils, i.e., belonging in the sections "Spira" or "Retinaculum-Apertum" of the Pridham et al. (1958) classification. However, the converse of this was not necessarily true, since several species having a spiralled-spore apparatus had smooth spores. In no instance did Streptomycetes with straight to flexous sporophores have other than smooth spores.

In table 1 it may also be observed that a remarkably constant spore surface morphology existed in most of the species studied. In a few instances, e.g., with *Streptomyces albus* (Rossi-Doria emend. Krainsky) Waksman and Henrici, *Streptomyces diastaticus* (Krainsky) Waksman and Henrici, *Streptomyces diastatochromogenes* (Krainsky) Waksman and Henrici, *Streptomyces olivaceus* (Waksman) Waksman and Henrici, and *Streptomyces erythraeus*, it would appear that this conformity did not hold, since reference strains of these species were found to have spores of different morphological types. However, upon closer examination the disparity between strains received from various collections under the same species name proved to be attributable to major taxonomic differences. In the case of the groups of *S. albus*, *S. diastaticus*, and *S. erythraeus* cultures, for instance, such profound differences as spore color and sporophore morphology were observed to exist between strains, and it was obvious that misidentifications were responsible for all the anomalies.

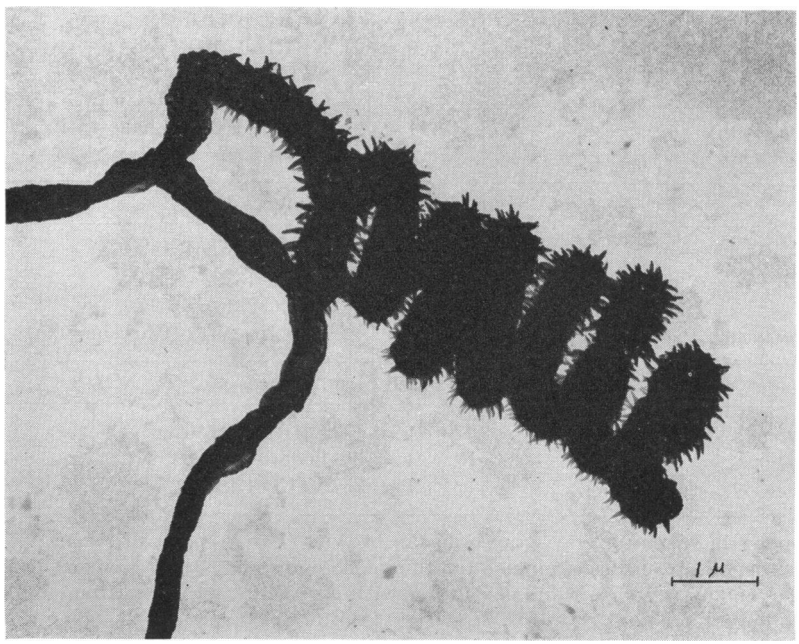

Figure 7. Spiral chains of ornamented spores of *Streptomyces viridochromogenes* (Lederle AS-361)

Minor variations in spore surface morphology between strains of some species have been observed, but these have been mostly a matter of degree. For instance, the amount of, or length of spines or hairs on the spores of certain organisms may differ from strain to strain, but never have the variations been sufficiently great as to alter the general spore morphological type.

The spores themselves have shown considerable diversity of form among the different species ranging from globose to elliptical to elongate or cylindrical. Although their size and shape has been found quite uniform in strains of some Streptomycetes, these features are not generally constant enough to provide reliable taxonomic criteria. Both the size and shape of spores can be modified in the electron microscope, the amount depending upon the intensity of the electron beam. Furthermore, the position of spores on the sporophore can be an influencing factor in their form and dimensions. Frequently, the terminally located spores are smaller and more uniform in size and shape than those more basally located; the basal spores often appear enlarged and misshapen.

It has been suggested that the nutritional environment may influence the surface configuration of Streptomyces spores. Lechevalier and Tihonienko (1960), in a study of two spiny-spored species on several defined and organic media, found that variation in the appearance of the spores occurred from one medium to another affecting the clarity with which spines could be demonstrated. Hence, it was of interest to us to determine whether morphological differences were produced by any of the media used in the present study. Several species representing the various spore morphological types were compared on the three agar media—yeast-malt, Waksman's starch, and tomato paste oatmeal. Insofar as could be observed there were no significant modifications in spore morphology of cultures brought about by these cultivation media.

DISCUSSION

In our experience, spore surface morphology as a taxonomic criterion is most valuable in two of the groups of Streptomycetes, namely those with spores in gray to gray-brown to brown shades and to a more limited extent, those with pinkish-cinnamon to pinkish-tan shades. Especially fortunate is the diversity of morphological types found in the larger gray to brownish-spored group. Since all of the four basic spore morphological types are present in this group, it can be conveniently divided into as many taxonomic sections. We have not considered it advisable, however, to make further subdivisions on the basis of the length of spore appendages, as was recommended by Ettlinger et al. (1958), because of the variability in this regard which we have encountered between strains of some species.

Ornamentation of spores among the pinkish-cinnamon to pinkish-tan species has been observed quite rarely and has always been of the spiny type. In *S. erythraeus* of this spore color group the spines not only cover the spores, but likewise appear prominently on the intercalary sheath between the often widely separated spores (figure 5). This has also been illustrated by Dolezilova, Vanek, and Kralik (1959). Spiny spores and sheaths have similarly been observed in strains of *S. purpurascens* of this same spore color group.

The taxonomic value of spore surface configuration in the other Streptomycetes does not appear to be quite so significant. In the blue to blue-green-spored members, all have spiny spores, and in the remaining spore color groups, all have had smooth spores. Despite the fact this is a limitation on the use of spore surface morphology as a taxonomic criterion, we do not find it a serious objection, because the majority of the species fall within the gray to brown or the pinkish-cinnamon to pinkish-tan spore color groups.

In general, we have noted that our observations of Streptomyces spore surface morphology have corroborated those of other investigators, and it would appear that the consensus of these workers endorses spore morphology as a constant feature within the species. In our present evaluation of this criterion we too have concluded that herein lies a reliable and convenient tool, made possible through electron microscopy, for systematizing this group of organisms.

ACKNOWLEDGMENT

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SUMMARY

Spore morphology of the Streptomycetes was studied by means of electron microscopy to

determine the reliability of this feature as a criterion for species differentiation in this genus. Approximately 600 cultures assignable to one or another of some 120 described species or varieties were included in the study. Twenty-five of the species were represented by an average of 14 strains each.

Spores of all the Streptomycetes studied were found to conform to one of four types—smooth, warty, spiny, or hairy. About one-third of the gray to brownish-spored species had either spiny, warty, or hairy spores; the remaining members of this spore color group were smooth-spored. All of the blue to blue-green-spored forms had spiny spores. Those having spore masses in white, or yellow to cream or buff shades had smooth-walled spores. All of the pinkish-cinnamon to pinkish-tan-spored group had smooth spores with the exception of *Streptomyces erythraeus*, *Streptomyces purpurascens*, and two undetermined species which had spiny spores.

The size and shape of spores in most species tended to be variable and appeared to be of limited usefulness for taxonomic differentiation. On the other hand, surface configuration of the spores was observed to be a remarkably constant species characteristic and promises to provide a reliable and useful taxonomic aid.

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