CONSTANCY OF CHARACTERISTICS IN THE STREPTOMYCETES[1]

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ABSTRACT

SANCHEZ-MARROQUIN, A. (University of Mexico, México D.F.). Constancy of characteristics in the streptomycetes. J. Bacteriol. **83**:1183–1192. 1962.—A total of 150 *Streptomyces* strains isolated from soil was studied during a 3-year period in regard to constancy and variation of the following characteristics: sporophore micromorphology, color of the aerial and substrate mycelium, surface configuration of the spores, assimilation of carbon compounds, production of H_2S and melanoid pigment, and reduction of nitrates. A remarkable constancy in the following characteristics was found: (i) sporophore micromorphology when only three of the seven morphological series of Pridham et al. were developed on tomato paste-oatmeal agar or yeast extract-malt extract agar; (ii) color of the aerial mycelium if only four fundamental colors are distinguished (white to cream or buff shades; yellow to orange or brown; pink to cinnamon, red or pinkish tan to lavender; and green to gray or blue); (iii) surface configuration of the spores divided in two types (smooth and warty to spinous or hairy); (iv) assimilation of five carbon compounds (arabinose, xylose, rhamnose, raffinose, and mannitol); (v) production of H_2S on Difco peptone-iron agar supplemented with 0.1% Difco yeast extract; and (vi) production of melanoid pigment on peptone agar, giving similar results to those of the H_2S test. Color of the substrate mycelium, size and shape of the spores, and reduction of nitrates should be used only as complementary data in the species descriptions, owing to their inconsistency and unreliability.

Various co-operative studies of taxonomic problems in the genus *Streptomyces* have been undertaken. Among them are those of the First International Co-operative Work on criteria used in characterization of streptomycetes (Küster, 1961), sponsored by the 7th International Congress of Microbiology, and two studies by the Subcommittee on Actinomycetes of the Committee on Taxonomy of the American Society for Microbiology. Only one of these studies has been published up to the present time (Gottlieb, 1961).

A number of investigators have extensively reviewed the many problems encountered in species differentiation (Hesseltine, Benedict, and Pridham, 1954; Waksman, 1957; Shinobu, 1958; Pridham, 1959; Routien, 1959; Gottlieb, 1961; Krasil'nikov, Nikitina, and Korenjako, 1961), especially those related to variation (Duggar, Backus, and Campbell, 1954; Backus, Duggar, and Campbell, 1954; Burkholder and Sun, 1954; Jones, 1954; Waksman, 1957).

It is the purpose of the present paper to estimate the frequency of some of these variations through a period of 3 years in 150 *Streptomyces* strains isolated from soil. Seven characteristics were considered as fundamental and studied particularly: sporophore micromorphology, color of the aerial and substrate mycelium, surface configuration of the spores, assimilation of carbon compounds, production of H_2S, development of melanoid pigment, and reduction of nitrates.

MATERIALS AND METHODS

Strains. The 150 *Streptomyces* strains were selected from approximately 1,000 strains isolated from soil in Mexico, Brazil, and Colombia; they were selected on the basis of sporophore morphology, color of the aerial mycelium, and C-assimilation patterns. They were stored on soil, from which they were seeded onto oatmeal agar and then, monthly, onto other media to seek variation. At the end of the first, second, and third years, observations were made after incubation at 28 C for 10 to 20 days for conidiophore production and color of mycelia; at 4 days for melanoid pigment development; at 10 days for electron microscopy of spores and

[1] A summary of this paper was presented before the 3rd National Congress of Microbiology, México D.F., October, 1960.

TABLE 1. *Composition of media (g/liter)*

Components	Oatmeal-agar	Yeast-extract agar	Tomato paste-oatmeal agar	Czapek	Potato-peptone glycerol	Glycerol-asparagine agar	Starch agar	Peptone agar	Glycerol-tyrosine agar
Potato					100				
Oat-meal	65		20						
Tomato paste			20						
Yeast extract		4						1	
Malt extract		10							
Soluble starch							10		
Glucose		4							
Glycerol (ml)					5	0.35			15
Asparagine									1
Peptone					2				
Typtone								1	
Tyrosine									0.5
K_2HPO_4				1	0.5	2.5	0.3		0.5
$MgSO_4$				0.5	0.5	0.3			0.5
KCl				0.5					
$FeSO_4$				0.01	0.01				0.01
$NaNO_3$				3			1		
$MgCO_3$							1		
NaCl				5	0.5	5	0.5	8.5	0.5
$CaCl_2$						0.1			
Sucrose				30					
Ammonium lactate						6.5			
Sodium asparaginate						3.5			
Agar	20	24	15	15	15	20	15	17	15
Distilled water		1,000		1,000	1,000	1,000	1,000	1,000	1,000
Tap water	1,000		1,000						

assimilation of carbon compounds; at 6 and 18 hr for H_2S production; and at 7 and 15 days for nitrate reduction.

Media. Oatmeal agar, tomato paste-oatmeal agar, yeast-extract agar, Czapek's agar, and peptone-glycerol-potato agar were employed for conidiophore production, and the same media plus glycerol-asparagine agar and soluble starch-salts agar for observation of mycelial colors. The development of melanoid pigment was studied on peptone agar and tyrosine-glycerol agar. The composition of each of these media is shown in Table 1.

To study carbon-assimilation patterns, the common solid and liquid media were employed (Pridham and Gottlieb, 1948; Benedict et al., 1955). The liquid media were used only when doubtful results were obtained after the first isolation, and at the end of the third year. Erlenmeyer flasks (250 ml) with 20 ml of Pridham and Gottlieb's synthetic liquid medium were utilized in these cases, adding 10 ml of the 1% chemically pure carbon compound solution filtered through Seitz filters, and 2 ml of a suspension of *Streptomyces* spores prepared following the usual procedure. Petri plates with the inoculated solid medium were incubated at 28 C for 10 days. Flasks with the liquid medium were incubated on a rotary shaker at 28 C for 4 days. Tubes with 10 ml of the same liquid medium were also used in this test run under static conditions, incubated at 28 C, and readings made at the end of 10 days.

Electron microscopy. The preparations were made following the usual spore-print technique in which Formvar-covered copper grids (200 mesh) were pressed against the sporulating surface of a 10-day-old culture on oatmeal-tomato paste agar, without fixing or shadowing. Most observations were made at a multiplication of 6,000 ×. In general, the recommendations of Zworykiiv et al. (1954) were followed.

Nitrate reduction. Comparative studies were

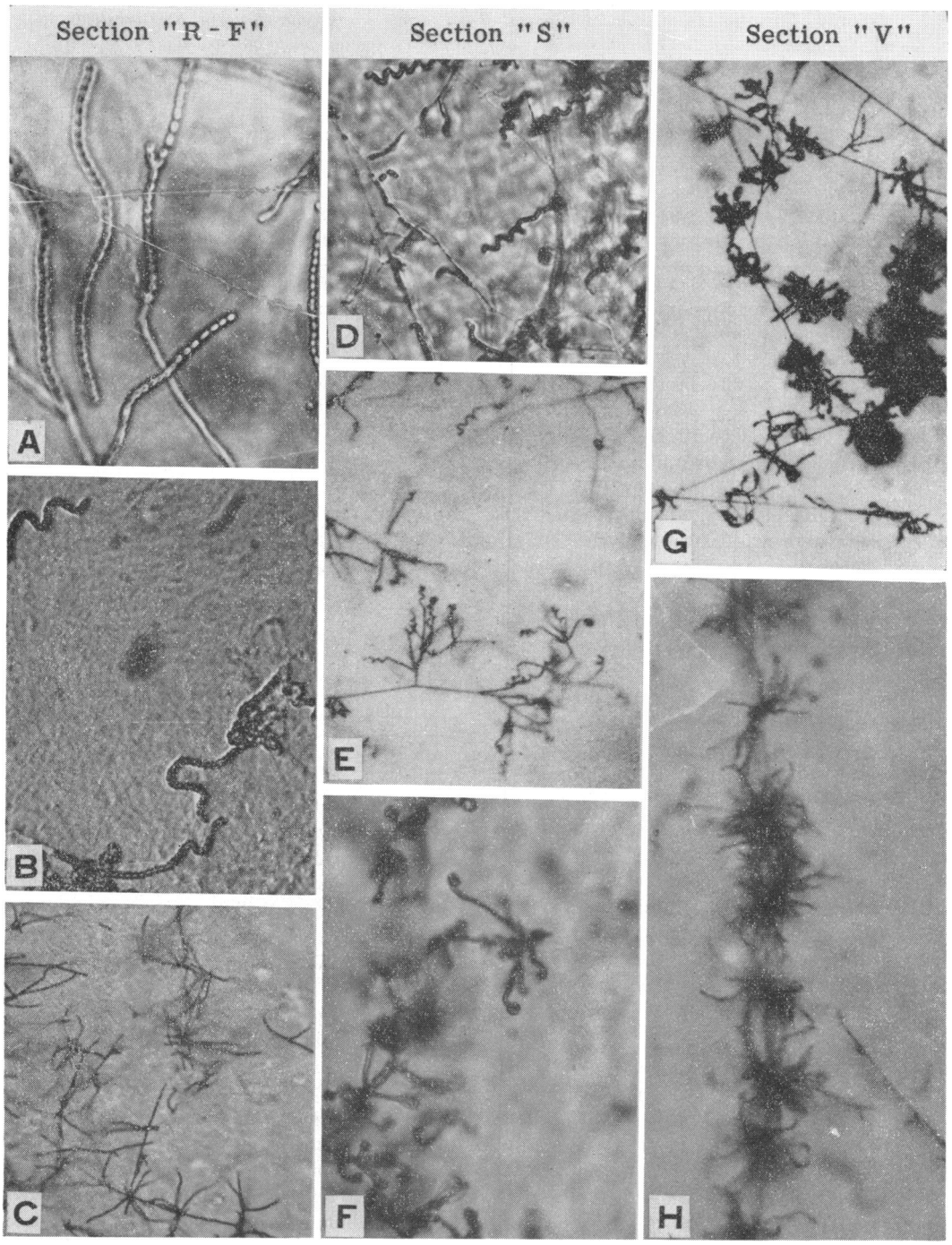

FIG. 1. *Conidiophore micromorphology. Section "R-F" (straight, flexuous, fascicle and hook nonverticillate forms): A, B, C. Section "S" (primitive spirals, open loops, open and closed nonverticillate spirals): D, E, F. Section "V" (verticillate forms, with or without spirals): G, H.*

TABLE 2. *Variation in micromorphological characteristics and color of aerial and substrate mycelium in 150 Streptomyces strains grown on tomato paste-oatmeal agar**

Observation	Conidiophores			Aerial mycelium								Substrate mycelium					
	R-F	S	V	W	Y	G	B	R	Gy	Br	L	W-Br	Y-O	Gy-Bl	R	G	B
1st Isolation	52	84	14	32	21	19	6	18	35	7	12	58	62	7	8	12	3
2nd Year	52	83	15	37	19	18	6	18	37	8	7	74	51	14	3	7	1
3rd Year	49	87	14	41	19	18	6	15	32	6	13	66	48	15	11	8	2

* Abbreviations: R-F = straight to flexuous; S = spirals; V = verticillate; W = white; Y = yellow; G = green; B = blue; R = pink to red; Gy = gray; Br = brown; L = lavender; Y-O = yellow to orange; Gy-Bl = gray to black

made on complex nitrate broth (peptone, 5 g; Difco meat extract, 3 g; KNO_3, 1 g; distilled water, 1000 ml) and synthetic nitrate broth (0.5% $CaCl_2$, 100 ml, in 900 ml of the following solution: K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KNO_3, 1 g; glucose, 10 g; distilled water, 900 ml). Results were read at the end of 7 and 15 days of incubation at 28 C. The presence of nitrites was estimated by means of the Ehrlich reagent. If no red color developed after addition of the reagent, a pinch of zinc dust was added. If nitrates were reduced to nitrite, the result was positive and the red color appeared. If no color appeared, the nitrate had been used completely or reduced to products beyond nitrite.

Production of H_2S. Difco peptone iron agar stabs, supplemented with 0.1% Difco yeast extract, were utilized and results read after 6 and 18 hr of incubation at 28 C.

Micromorphology. The Pridham, Hesseltine, and Benedict (1958) system was first followed; then the morphological types were reduced to only three (Fig. 1): "R-F" (Rectus-Flexibilis), "S" (Spira), and "V" (Verticillate).

RESULTS

Sporophore micromorphology and color of aerial and substrate mycelium. The variation in micromorphological characteristics and color of aerial and substrate mycelium in the 150 *Streptomyces* strains under study are shown in Table 2. A predominance of spiral forms over the R-F types was observed, and the R-F types were more common than the verticillate forms. Culture media showed no influence on the micromorphology of the conidiophores; therefore these data refer only to the tomato-paste agar, taken as representative. The constancy of the conidiophore appearance at the end of the third year is a remarkable feature. On the other hand, great discrepancies were found when a sharp distinction of the spiral forms (compact, loose, dextrorse, or sinistrorse) was attempted and also when subdivisions in the verticillate series were tried. If the seven types of sporophore morphology established by Pridham et al. (1958) were differentiated, over-all agreement was absolutely impossible, especially regarding the second, third, sixth, and seventh series, which are difficult to ascertain and which vary with the culture medium used.

TABLE 3. *Electron microscopy of the spore surface in 125 Streptomyces strains*

Color of aerial mycelium	Rough or warty		Spinous	Hairy	Smooth	
	1st yr	3rd yr	1st and 3rd yr	1st and 3rd yr	1st yr	3rd yr
White to buff or tan	0	2	0	0	32	30
Yellow	0	0	0	0	21	21
Brown	1	1	1	0	5	5
Gray to blue	2	2	6	0	27	27
Pink to lavender	0	0	4	0	26	26

Eight colors named in ordinary parlance were considered for the aerial mycelium color estimation and six for the substrate mycelium. More variations were observed in the white and red aerial mycelium series, especially in the first one; many of them changed to light gray, yellowish tan, and even pink. This occurred in both the tomato paste-oatmeal and yeast extract-malt extract media, which are considered best for this purpose. The changes were even more ap-

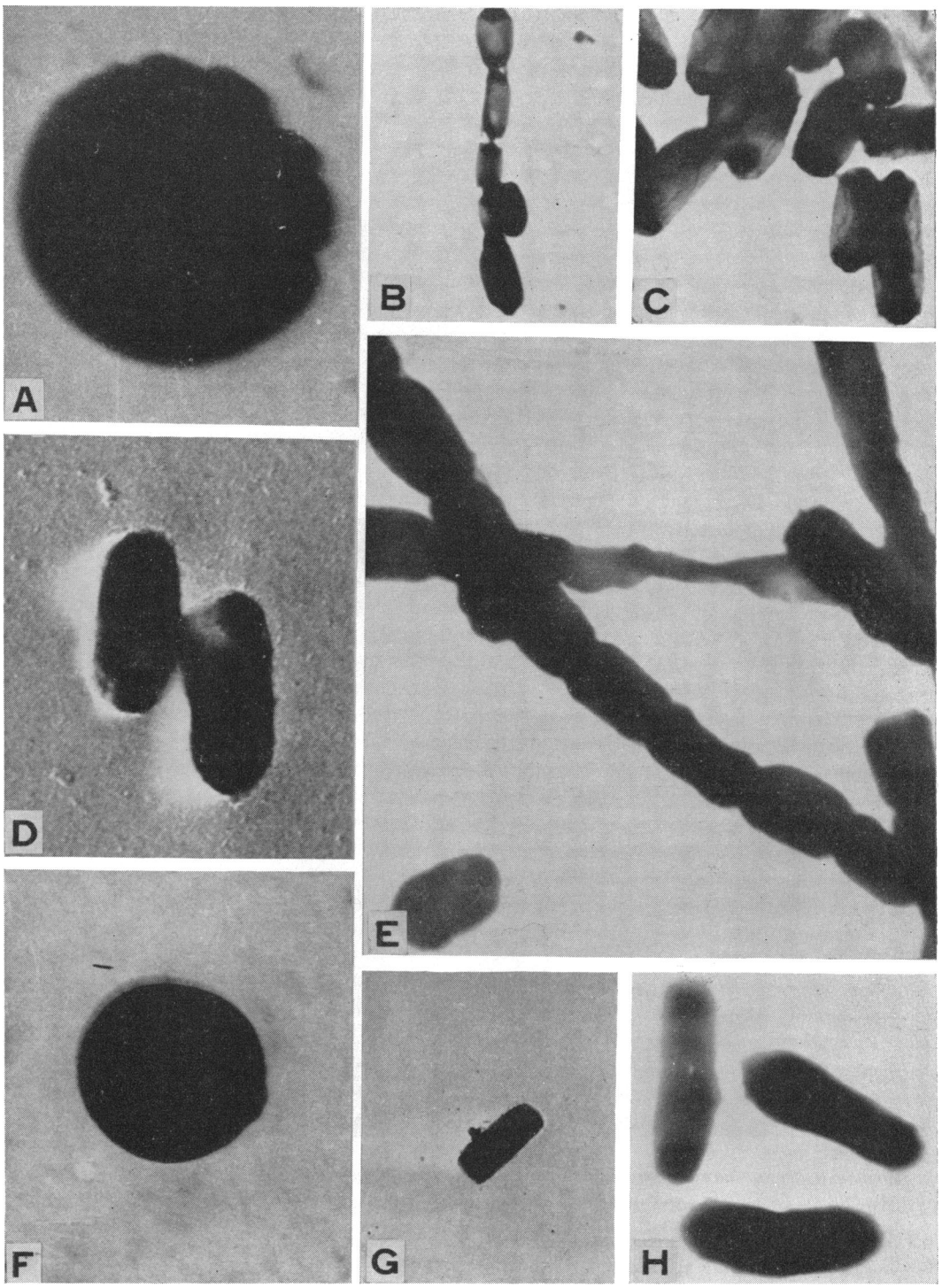

FIG. 2. *Surface configuration of the spores: smooth types. A, smooth spherical type developing a warty appearance. This and the smooth type shown in F were found in the same preparation; B, smooth types formed by fragmentation, with hyphal wall still visible. Later on, mature spores are released (C); D, mature, elongated spores with rounded ends, derived by fragmentation from "R-F" sporophores as shown in E; G, smooth cylindrical types, which usually coexist with ellipsoidal or elongated forms; H, rod-like and irregular forms.*

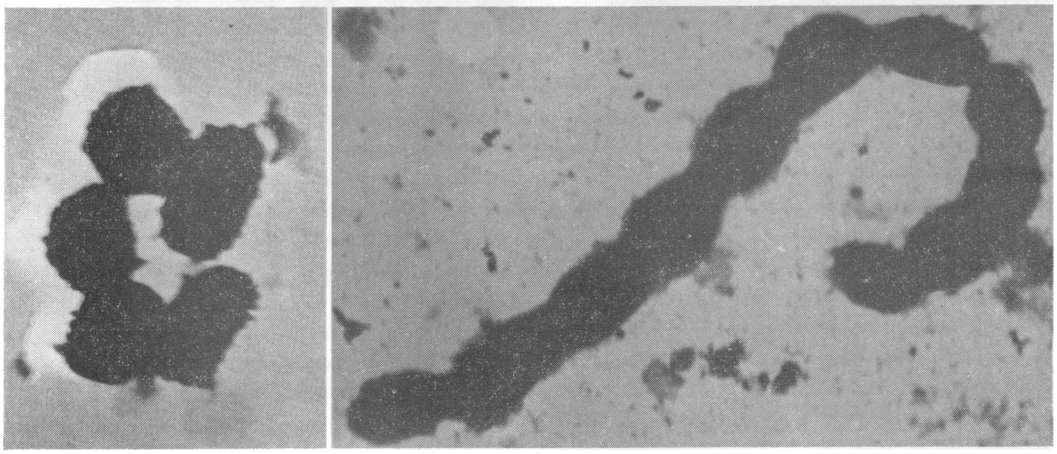

FIG. 3. *Surface configuration of the spores: warty types*

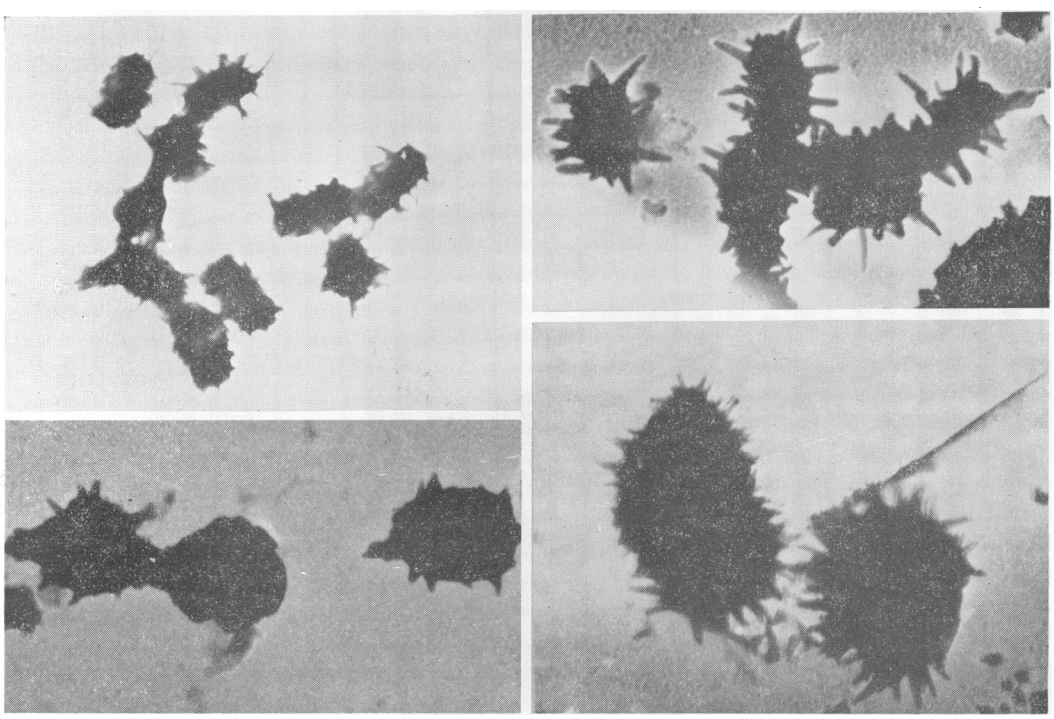

FIG. 4. *Surface configuration of the spores: spinous types. These forms as well as those shown in Fig. 3 (warty) and the hairy-like outgrowths should be included in just one: the prickly type.*

parent in glycerol-asparagine and soluble starch media. The latter results are not reported in the present study.

Surface configuration of the spores. Spores were differentiated into five types according to their colors (first isolation): 32 white to cream or buff; 21 yellow; 7 brown; 35 gray to blue; and 30 pink, pinkish, cinnamon, red, or lavender (Table 3).

Of the four spore surface types reported (Flaig and Kutzner, 1954; Flaig, Küster, and Beutelspacher, 1955; Kutzner, 1956; Tressner,

Davies, and Backus, 1961), only three were observed in 125 strains: smooth (Fig. 2), warty (Fig. 3), and spinous (Fig. 4), with a predominance of the smooth type. The hairy type was not found in these studies.

Differentiation of oval, round, or cylindrical shapes in the smooth series was not a constant characteristic; the different forms may appear together in the same preparation.

TABLE 4. *Carbon-assimilation patterns in Pridham and Gottlieb's solid medium*

Carbon source	1st isolation			3rd yr			Per cent doubtful (±)	
	−	±	+	−	±	+	1st isolation	3rd yr
L-Arabinose	3	2	38	3	2	38	4.6	4.6
D-Xylose	4	1	38	4	1	38	2.3	2.3
D-Glucose	0	1	43	0	0	43	2.3	0
D-Galactose	12	5	26	14	8	21	11.6	18.6
Rhamnose	12	2	29	13	1	29	4.6	2.3
Sucrose	18	5	20	20	6	17	11.6	13.9
Maltose	6	4	33	5	7	31	9.2	16.2
Lactose	10	2	31	7	8	28	4.6	18.6
Raffinose	22	3	18	22	1	20	6.9	2.3
Inulin	6	8	29	7	9	27	18.6	20.9
D-Mannitol	18	2	23	18	2	23	4.6	4.6
D-Dulcitol	42	0	1	43	0	0	0	0
i-Inositol	34	2	7	32	6	5	4.6	13.9
D-Sorbitol	13	17	13	15	14	14	39.5	32.5

Carbon-assimilation tests. The results of carbon-assimilation tests on Pridham and Gottlieb's solid medium are presented in Table 4. Even though 14 carbon compounds were used in the tests, results indicate that the total number of compounds could be reduced to five or even less; most consistent results were shown by: arabinose, xylose, rhamnose, raffinose, and mannitol. Glucose was assimilated by all of the strains tested, whereas D-dulcitol was not. D-Galactose, sucrose, maltose, and inulin gave a high percentage of doubtful results, both in first isolation and at the end of the third year.

Strains giving doubtful results on the 14 compounds tested were also studied on Pridham-Gottlieb liquid medium, with and without agitation (Table 5). Generally speaking, the liquid media, with shaking, gave the fewest doubtful results.

NO_3 reduction, production of H_2S, and melanoid pigment. Results of these tests are shown in Table 6. Considerable variability in NO_3 reduction by the 150 strains was apparent. On the contrary, H_2S production and development of melanoid pigment showed only a very slight variation during the 3-year period of observation.

TABLE 5. *Behavior of doubtful strains in Pridham-Gottlieb solid and liquid media**

Carbon source	First isolation			Third year		
	Solid medium ±	Liquid medium		Solid medium ±	Liquid medium	
		Static	Agitation		Static	Agitation
L-Arabinose	2	2+	2+	2	2±	2+
D-Xylose	1	1+	1+	1	1±	1+
D-Glucose	1	1+	1+	1	1+	1+
D-Galactose	5	4±, 1+	3+, 2−	8	4+, 4±	4+, 4−
Rhamnose	2	1±, 1+	2+	1	1−	1+
Sucrose	5	2+, 3−	5+	6	3+, 3±	6+
Maltose	4	4−	4+	7	4±, 3−	3+, 4−
Lactose	2	2+	2+	8	6±, 2−	4+, 4±
Raffinose	3	2±, 1−	3−	2	2±	2+
Inulin	8	6−, 2±	3+, 5−	9	6+, 3−	5+, 3±
D-Mannitol	2	1±, 1+	2+	2	2+	2+
D-Dulcitol	2	2−	2−	4	4−	4−
i-Inositol	2	2−	2−	6	6±	4±, 2−
D-Sorbitol	17	14±, 3+	12+, 5±	14	10±, 4−	8+, 6±

* Symbols: ±, doubtful; +, assimilation; −, no assimilation.

TABLE 6. *Reducion of nitrate and production of hydrogen sulfide and melanoid pigment*

Observations	NO_2 in NO_3-broth				H_2S		Melanoid pigment			
	Synthetic		Organic		+	−	Peptone agar		Tyrosine-glycerol	
	+	−	+	−			+	−	+	−
1st Isolation..	93	67	115	35	65	85	66	84	59	91
2nd Year...	81	69	98	52	66	84	66	84	61	89
3rd Year...	72	78	87	63	66	84	68	82	58	92

DISCUSSION

For differentiation of the micromorphological characteristics of the conidiophores, the Pridham et al. (1958) system was only partially followed. For convenience, the straight, flexuous, fascicle, and hook types were assembled together in section "R-F" (Rectus-Flexibilis), sympodic or monopodic nonverticillate forms. Sections Retinaculum-Apertum and Spira (primitive spirals, open loops, open spirals, and closed spirals) were reduced to only one nonverticillate form, and named "S" (Spira), regardless of the spirals being loose or compact. Finally, sections Monoverticillus, Monoverticillus-spira, Biverticillus, and Biverticillus-spira were reduced to "V" forms (verticillate series) with or without spirals. I think that in this way mistakes in identification of these forms will be considerably reduced.

It was considered essential to select the best media for growth and sporulation. In this regard, tomato paste-oatmeal agar and yeast extract-malt extract agar showed the best results. Only when scant growth was evident were other media used (mainly glycerol-asparagine or peptone-potato agar).

Shinobu (1958) recommended ammonium-Czapek agar, glucose-asparagine agar, and glycerine-starch-glutamine agar as the best media for morphological studies. He considered branching of the aerial mycelium and the formation of whorls and spirals as two of the most important characteristics. He also found that the various spiral forms may coexist, and are, therefore, not useful characteristics for taxonomic studies, except when certain spiral forms are specific and dominant.

Regarding mycelial color, there was a complete lack of agreement on enlarging the color range or in trying to distinguish the green to blue or yellow to brown shades.

Pridham et al. (1958) considered nine colors as fundamental; Burkholder and Sun (1954), seven; Ettlinger, Corbaz, and Hütter (1958), six; and Hesseltine et al. (1954), five. Since estimation of color is a subjective process, reduction in the basic color range should be a matter of international agreement.

In differentiating closely related colors, we have been unable to get good agreement or evaluation even with the use of the ISCC-NBS or Mertz and Paul terminology.

On this basis, we propose to reduce the color ranges for these studies to only four: (i) white to cream or buff; (ii) yellow to orange or brown; (iii) pink to pinkish cinnamon, red, or lavender; and (iv) green to gray or blue. This simple color designation would lead to a better judgement of this test and to a reduction of what Gottlieb calls "human variation." Thus, data derived from periodic observation of spore masses would be of some help to taxonomists. On the contrary, color of the vegetative or substrate mycelium does not seem to be a reliable tool for taxonomic purposes, on account of the great discrepancies encountered. Perhaps the value of this characteristic has been overemphasized. I believe it might have a relative value when a special synthetic medium is used and the color is seen very distinctly.

All of the white and yellow strains in this study had smooth surface spores, in agreement with recent reports (Tressner et al., 1961), while brown and gray to blue strains exhibited all three types of spore surface configuration, with a predominance of the smooth types. The pink to red or violet types were usually smooth or spinous. Baldacci (1958) maintains that the series described by Baldacci, Spalla, and Grein (1954) are uniform as far as the spore shape is concerned, but not on a surface-configuration basis. According to my data, the surface configuration, rather than the shape, is a more constant characteristic.

Differentiation of a simple rough type is sometimes a little artificial, because it may derive from a smooth type; or it may be considered a result of a vacuum effect of the electron beam (Enghusen, 1955). No difficulty was found in the separation of the other types. However,

we agree with Krasil'nikov et al. (1961) that only two types should be considered: smooth and prickly. It seems to be a fact that surface configuration of the spores is a reliable and remarkably constant characteristic and therefore a very useful taxonomic aid.

A similar conclusion could be derived from the results obtained in the carbon-assimilation tests. Data presented here lead to the following conclusions: (i) when essential differences in assimilation patterns appear and the number of doubtful results increases, the selective efficiency of the test is reduced to a point, eventually, where it could not be used for taxonomic purposes (Zähner and Ettlinger, 1957); (ii) in comparing the results obtained with maltose and sucrose, there is a clear-cut difference under static and submerged conditions of growth in liquid vs. solid media, whereas no essential differences appeared in the other compounds tested; (iii) if the carbon compounds giving 25% doubtful results are eliminated, then the most efficient compounds would be arabinose, xylose, rhamnose, lactose, raffinose, mannitol, and inositol; (iv) glucose and dulcitol could be eliminated on account of the wide utilization of the former and no assimilation of the latter; (v) lactose and inositol assimilation, being the most variable, could also be suppressed. Therefore, the carbon-assimilation patterns can be reduced, as Zähner and Ettlinger (1957) suggested, to only five compounds: arabinose, xylose, rhamnose, raffinose, and mannitol. Generally speaking, liquid media, under shaking conditions, give the fewest doubtful results.

The constancy of results in the assimilation of these five compounds seems to be an acceptable characteristic which could be used, together with micromorphological and selected biochemical tests, in the classification of the streptomycetes.

Pridham (*personal communication*) has suggested that the number of carbon compounds could be reduced to only three, whereas Shinobu (1958) stated that ten are essential.

These results show that nitrate reduction is not a consistent test, either in synthetic or organic media; it appears to be of no great value in *Streptomyces* characterization. On the other hand, production of H_2S is a very constant feature, as shown in several recent reports (Ettlinger et al., 1958; Tressner and Danga, 1958; Gottlieb, 1961). Accordingly, it seems logical to include this test as a reliable criterion for taxonomic differentiation of the streptomycetes, in conjunction with or instead of the melanoid-pigment production test with which it shows a remarkable parallelism. For this last purpose, peptone agar seems to be a good medium.

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