est expense that might be encountered is represented by the large vacuum pump required to operate the system across a critical orifice at 28.3 L per min. A smaller pump would be suitable if properly ballasted to maintain a constant rate of flow through the system.

ACKNOWLEDGMENT

The guidance of J. M. DallaValle is gratefully acknowledged; without his original plans from which this work stemmed, none of these studies could have been carried out.

SUMMARY

A method is presented for the evaluation of aerial disinfectants. This method, properly applied, is capable of yielding reproducible results expressed in a manner which can be applied universally. The equipment required is relatively inexpensive and is fabricated from generally available materials.

The modifying effect of relative humidity on the activity of aerial disinfectants is taken into consideration in this system, and compounds are offered as standards of reference in light of this effect.

Methods are given for the production of a standard bacterial aerosol, as are the details of equipment for diluting this aerosol, mixing it with chemical vapors, and sampling the resultant mixture.

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A Broadened Concept of the Characteristics of *Streptomyces hygroscopicus*

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It is becoming increasingly apparent that marked variability abounds in a great many of the species of *Streptomyces* and, as a result of the indiscriminate granting of species status to numerous variants of already defined species, a needlessly complex taxonomic system has arisen. Burkholder and Sun (1954) have discussed criteria for speciation in *Streptomyces* and have stressed the need for a system of convenience in which a relatively few named species groups would be established. Hesseltine *et al.* (1954) emphasized the need for uniformity in methods of study and of reporting data relative to taxonomic studies, while Jones (1954) pointed out the need for a better understanding of the organisms themselves before progress can be made in comprehending variability in *Streptomyces*. Backus *et al.* (1954), in their study of variability in *S. aureofaciens*, and Duggar *et al.* (1954), studying the same organism as well as other species of *Streptomyces*, have made clear the necessity of examining a large assemblage of related forms as a prerequisite to establishing species boundaries. Our continuing interest in the fundamental problem of speciation in the genus *Streptomyces* prompted us to undertake the present study of variability in *S. hygroscopicus* (Jensen) (Waksman and Henrici) in an attempt to delimit more clearly the species boundaries of this organism.

While it is recognized that a majority of the cultures involved in this study are capable, under specific conditions, of elaborating one or more products which possess substantial and varied antimicrobial activity, it is beyond the province of this paper to discuss such products or activities. Furthermore, it is felt that neither the capacity of a culture to produce such products nor the nature of such products themselves

is of controlling significance in species differentiation in the case of *S. hygroscopicus*.

Experimental Methods

A detailed study was made of 12 soil isolates which were selected as representative of the range of variability found in an orginal group of several hundred isolates determined to be *S. hygroscopicus*. Compared with and supplementing these were four type strains of *S. hygroscopicus* (ATCC #10976, Baarn Strain, NRRL B-1503 and NRRL B-1346). Also included were type cultures of two other described species, namely *S. endus* Strain NRRL B-2339 (Gottlieb *et al.*, 1951) and *S. platensis* Strain NRRL B-2364 (McGuire, 1954), both of which have been observed from time to time to exhibit all of the basic characteristics of the *S. hygroscopicus* group.

Table 1. *Media employed for cultural characterization of Streptomyces hygroscopicus strains*

Asparagine-dextrose meat extract agar (pH 6.5)
Asparagine-dextrose meat extract agar (pH 5.0)
Bennett's agar
Calcium malate agar
Carrot plugs
Cellulose (filter paper in Czapek's solution)
Corn Steep liquor agar
Czapek's-Dox agar
Czapek's-Dox mannitol agar
Czapek's-Dox starch agar
Emerson's agar
Gelatin
Krainsky's dextrose agar
Litmus milk
Potato dextrose agar
Potato plugs
Sabouraud's maltose agar
Waksman's glucose agar
Waksman's nutrient agar
Waksman's starch agar
Yeast extract agar

Spore suspensions of each of the 18 selected strains were used to inoculate the 21 differential media (table 1) which were used in the cultural and morphologic studies of the group; the agar media were streaked in a cross-hatched fashion. After incubation for 14 days at 28 C, the cultural and morphologic features of each strain, together with certain physiologic reactions, were studied and recorded (tables 2 and 3).

The pattern of utilization, by these strains, of carbon- and nitrogen-furnishing compounds was determined by methods adapted from those of Pridham and Gottlieb (1948). The various carbon and nitrogen sources employed, listed in tables 4 and 5 respectively, were sterilized by filtration. Each compound was added aseptically at the rate of 1 per cent to a sterile, melted, basal agar medium and dispensed into sterile screw-capped tubes and slanted. Aqueous suspensions of doubly washed spores from two-week-old agar slants were used to inoculate the tubes.

Results

Through a critical study of all the selected strains on the various differential media, it was discovered that all of the cultures had certain fundamental characteristics in common. Most underlying of these are the following: (1) The sporiferous appendages arise as short side branches of main hyphae and generally terminate in tight spirals of two to many turns (figure 1); frequently, there is also evident a tendency for clustering of the sporiferous structures. (2) On media which promote abundant sporulation, all strains studied were found to produce spores which were a

Table 2. *Distribution of hygroscopic character as observed on a variety of agar media*

	Asparagine-Dextrose Meat Extract	Acid Asparagine Dextrose Meat Extract	Czapek's	Potato Dextrose	Waksman's Starch	Waksman's Glucose	Waksman's Nutrient	Bennett's	Emerson's	Krainsky's Dextrose	Sabouraud's Maltose	Corn Steep Liquor	Calcium Malate	Czapek's Starch	Czapek's-Dox Mannitol	Yeast Extract
Streptomyces hygroscopicus ATCC #10976	+							+		+	+					+
S. hygroscopicus Baarn Strain	+	+						+			+	+				
S. hygroscopicus NRRL B-1346	+	+						+		+		+				+
S. hygroscopicus NRRL B-1503	+	+			+					+			+		+	+
S. endus NRRL B-2339	+	+			+			+		+		+		+	+	+
S. platensis NRRL B-2364	+	+			+			+		+		+				+
S. hygroscopicus A-9822														+		
S. hygroscopicus AA-214	+	+			+			+		+		+	+			+
S. hygroscopicus AA-398				+				+				+				
S. hygroscopicus AB-623					+			+								
S. hygroscopicus AB-965	+				+			+				+				
S. hygroscopicus T-1961	+	+			+			+		+		+				+
S. hygroscopicus T-3580	+	+		+				+		+						+

TABLE 3. *Distribution of diffusible pigment as observed on various agar media*

	Waksman's Starch	Calcium Malate	Yeast Extract	Sabouraud's Maltose	Waksman's Glucose	Potato Dextrose	Corn Steep Liquor
Streptomyces hygroscopicus NRRL B-1503	Buff; light	Brownish; light	Yellowish-brown; light				
S. hygroscopicus NRRL B-1346		Yellowish; light					
S. hygroscopicus ATCC #10976					Yellowish; light		
S. hygroscopicus Baarn Strain							
S. platensis NRRL B-2364	Yellowish; light	Yellowish; light	Yellowish-brown; light	Yellowish; light		Vinaceous; light	Yellowish; light
S. endus NRRL B-2339		Brownish; light	Yellowish; light				
S. hygroscopicus A-33	Yellowish; moderate	Yellowish; moderate	Yellowish; light	Yellowish; light			Yellowish; light
S. hygroscopicus A-9935			Yellowish-brown; light				
S. hygroscopicus AA-214	Yellowish; light	Vinaceous; abundant			Yellowish; light	Vinaceous; light	Brownish; light
S. hygroscopicus AA-398				Vinaceous; light			
S. hygroscopicus AB-623	Brownish; light		Brownish; light	Brownish; abundant			Brownish; light
S. hygroscopicus AB-965	Yellowish; moderate			Yellowish; abundant	Yellowish; moderate		Yellowish; moderate
S. hygroscopicus AC-365		Yellowish; moderate					
S. hygroscopicus T-1961	Yellowish; moderate	Yellowish; light	Yellowish-brown; moderate	Yellowish-brown; abundant	Yellowish; moderate	Vinaceous; light	Yellowish; light
S. hygroscopicus T-3580	Yellowish; moderate	Yellowish; abundant	Yellowish; abundant	Yellowish-brown; abundant	Yellowish; moderate	Yellowish; light	Brownish; moderate

TABLE 4. *Variation in carbon source utilization by Streptomyces hygroscopicus strains*

	Number of Strains		
	Positive		Negative
	Good	Poor	
Glycerol	18		
Mannose	17	1	
D (+) Trehalose	17	1	
Mannitol	16	2	
D (+) Levulose	15	3	
D (+) Xylose	8	10	
Raffinose	15	2	1
Na citrate	8	8	2
Lactose	7	10	1
Sucrose	9	4	5
Sorbitol	8	5	5
D (−) Arabinose	6	6	6
L-Rhamnose	5	4	9
Erythritol	3	5	10
Inulin	1	2	15
Sorbose			18

TABLE 5. *Variation in nitrogen source utilization by Streptomyces hygroscopicus strains*

	Number of Strains		
	Positive		Negative
	Good	Poor	
Alanine	18		
Aspartic acid	18		
Histidine	15	3	
Glutamine	16	1	1
Glycine	16	1	1
Arginine	15	2	1
Proline	14	2	2
Valine	12	4	2
Leucine	11	5	2
Phenylalanine	9	7	2
Urea	8	7	3
Methionine	6	9	3
Glutamic acid	10	4	4
$NaNO_3$	5	5	8
$(NH_4)_2HPO_4$	1	3	14
$NaNO_2$			18

brownish-gray color *en masse* (*Mouse Gray* to *Benzo Brown* of Ridgway, 1912). (3) All strains studied developed to some degree, on one or more media which promotes sporulation, the characteristic moist, glistening, dark hygroscopic patches in the aerial mycelium which led to the designation of the species as *S. hygroscopicus*. This latter feature, while not exclusively associated with this species, being present also in *S. aureofaciens* and others, is readily observable and serves as one of the chief aids in the initial recognition of possible members of this group. Turning now from the features which have been found to be relatively constant, let us consider the range of variation which has been evident in this group of cultures in regard to spore morphology, growth habits, pigment production, and certain other physiologic reactions on a variety of media.

Spores were found to vary in shape from globose to elliptic to isodiametric cells with truncate ends. Spore sizes ranged for the most part from 0.6 to 1.2 μ in globose or isodiametric-truncate types to 0.6 to 0.9 by 0.9 to 1.8 μ in elliptic types. Spores of some strains tend to adhere together in short chains, frequently still in the form of a partial spiral turn, when mounted in water, while those of other strains tend to break apart more readily.

Growth of most strains on potato plugs was vigorous, with sporulation in shades of *Mouse Gray* (Ridgway, 1912); similar growth and sporulation was observed on carrot plugs, but more variability between strains occurred.

The ability of this group of organisms to decompose cellulose, as determined by growth on filter paper in Czapek's solution, was extremely variable. About 50 per cent of the strains studied gave positive results. This variability was particularly evident in the type *S. hygroscopicus* strains. The Baarn culture collection strain showed good utilization, while the two NRRL strains gave intermediate reactions and the ATCC strain failed to give any positive response.

Gelatin was liquified to some extent by all of the cultures studied, and completely by a majority of them.

Behavior of both type cultures and soil isolates on litmus milk was so variable as to be of no possible value in species characterization. Considering the group as a whole, growth was fairly good and some degree of peptonization was evident in about 60 per cent of the strains studied.

A further insight into the range of variability which the diverse strains of *S. hygroscopicus* may encompass is obtained by a consideration of the characteristics which they display on a group of agar media commonly employed in studies of members of this genus. Among the features illustrative of such variation are: (1) the amount and nature of the substrate growth including color, marginal character, and general texture; (2) the amount of aerial mycelium and sporulation developed, its general nature and pattern of distribution on the substrate thallus; (3) presence or absence of moist, black, hygroscopic areas and their distribution on the colony; (4) presence or absence of diffusible

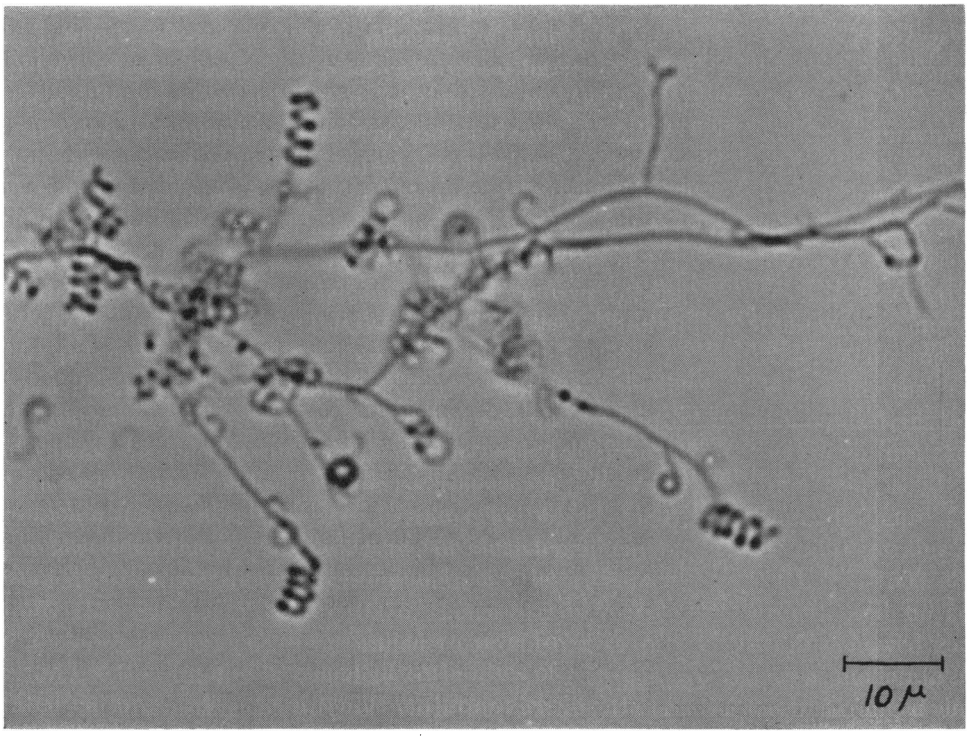

FIG. 1. Sporiferous appendages of *Streptomyces hygroscopicus*

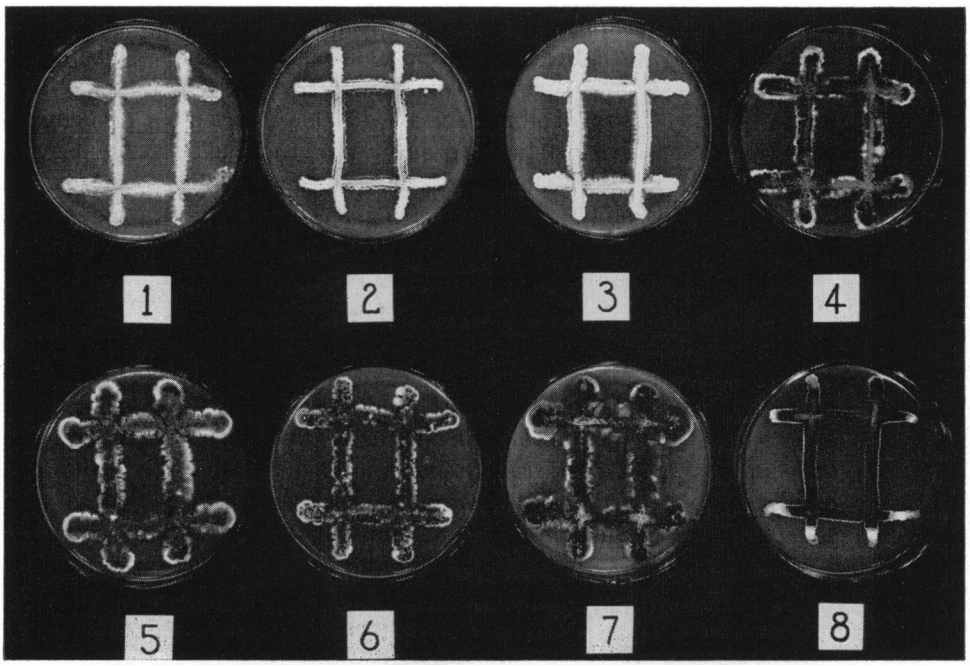

FIG. 2. Cultural features of six type cultures plus two soil isolates on Waksman's starch agar: (1) *Streptomyces hygroscopicus* ATCC #10976. (2) *S. platensis* NRRL B-2364. (3) *S. hygroscopicus* soil isolate A-33. (4) *S. hygroscopicus* NRRL B-1346. (5) *S. hygroscopicus* Baarn strain. (6) *S. endus* NRRL B-2339. (7) *S. hygroscopicus* NRRL B-1503. (8) *S. hygroscopicus* soil isolate T-1961.

pigments, including a consideration of the amount and color of such substance if present; (5) reverse color, a character associated largely with the color of the substrate growth but modified by the nature of the medium involved and the time of observation; and (6) presence or absence of exudates on the colony surfaces and the color of such exudates if present.

The hygroscopic feature associated with *S. hygroscopicus* is not developed constantly on all agar media, and the degree to which it is expressed on a particular medium also may vary somewhat from time to time. Table 2 shows the distribution pattern of the hygroscopic characteristic on various agar media as displayed by several cultures, both culture collection types, and soil isolates. It may be observed that some, such as *S. hygroscopicus* Strain NRRL B-1503, *S. endus*, *S. platensis*, and soil isolate Strain AA-214, display the feature on several media, whereas others, such as Strains A-9822, AB-623 and AA-398, show it only on a few.

The production of diffusible pigment on agar media by the various strains of the *S. hygroscopicus* group is also a variable feature as is shown in table 3. Pigments ranged from shades of yellowish to brownish or vinaceous. Certain soil isolates (Strains T-1961, T-3580), as well as the *S. platensis* type, produced pigment on many media; whereas other strains (*S. hygroscopicus* NRRL B-1346, ATCC #10976, Baarn Strain, A-9935, AA-398, and so on) either failed to produce it or produced it only on a few media.

An interesting picture of variation within the *S. hygroscopicus* complex can be obtained also by a comparison of the gross cultural characteristics exhibited by different strains on various agar media. To illustrate this, several strains were compared on three differential media: Waksman's starch agar, Sabouraud's maltose agar, and calcium malate agar.

In figure 2, the growth habits and other cultural features of the six types plus two soil isolates are shown on Waksman's starch agar. This medium is generally good for growth and sporulation of most strains of *S. hygroscopicus*; however, it does produce a differential response by some strains. The ATCC strain, the two soil isolates, and the *S. platensis* type show a somewhat restricted growth, while the other type strains of *S. hygroscopicus* and *S. endus* show a spreading habit. The cultures have been arranged in their approximate order of increasing sporulation and degree of hygroscopicness. Thus, it may be seen that the first three strains are lightly sporulating, with the hygroscopic character absent or poorly developed, while increasing sporulation and hygroscopicness is shown in the remaining cultures, becoming most pronounced in the last culture (soil isolate T-1961).

The cultural responses of the six types plus two other soil isolates on Sabouraud's maltose agar are shown in figure 3. This medium is excellent for growth of most strains of *S. hygroscopicus*; however, the NRRL B-1503 strain is an exception, with only thin growth and no sporulation produced. The remaining strains showed good to exceptional growth with sporulation moderate to sparse. Again the cultures have been arranged in their general order of increasing sporulation. In the NRRL B-1346 and ATCC strains

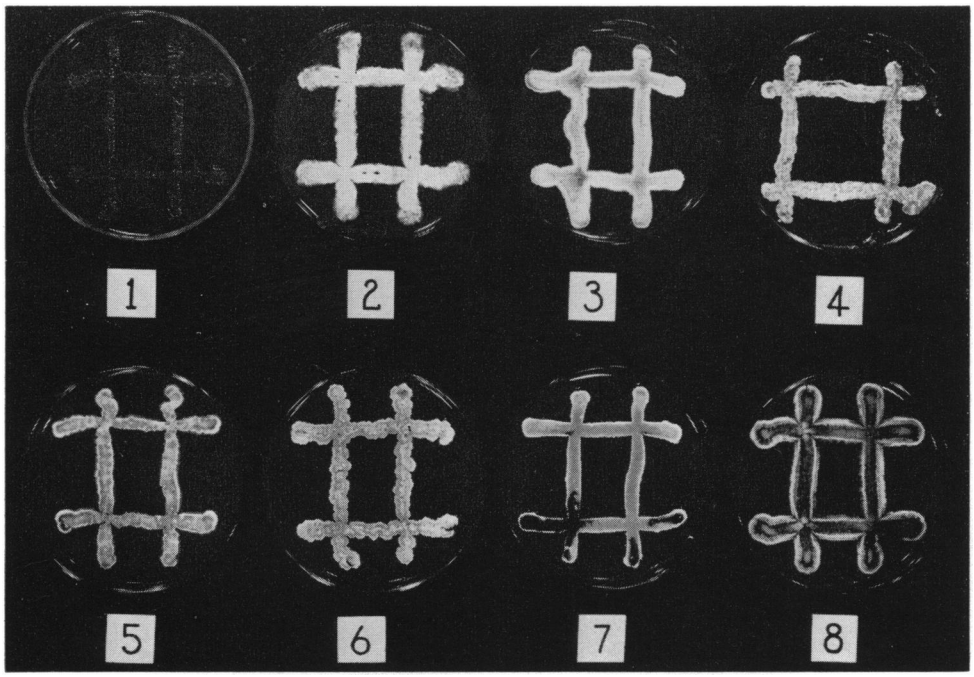

FIG. 3. Cultural responses of six type cultures plus two soil isolates on Sabouraud's maltose agar: (1) *Streptomyces hygroscopicus* NRRL B-1503. (2) *S. hygroscopicus* NRRL B-1346. (3) *S. hygroscopicus* ATCC #10976. (4) *S. platensis* NRRL B-2364. (5) *S. endus* NRRL B-2339. (6) *S. hygroscopicus* soil isolate AB-623. (7) *S. hygroscopicus* soil isolate AB-965. (8) *S. hygroscopicus* Baarn strain.

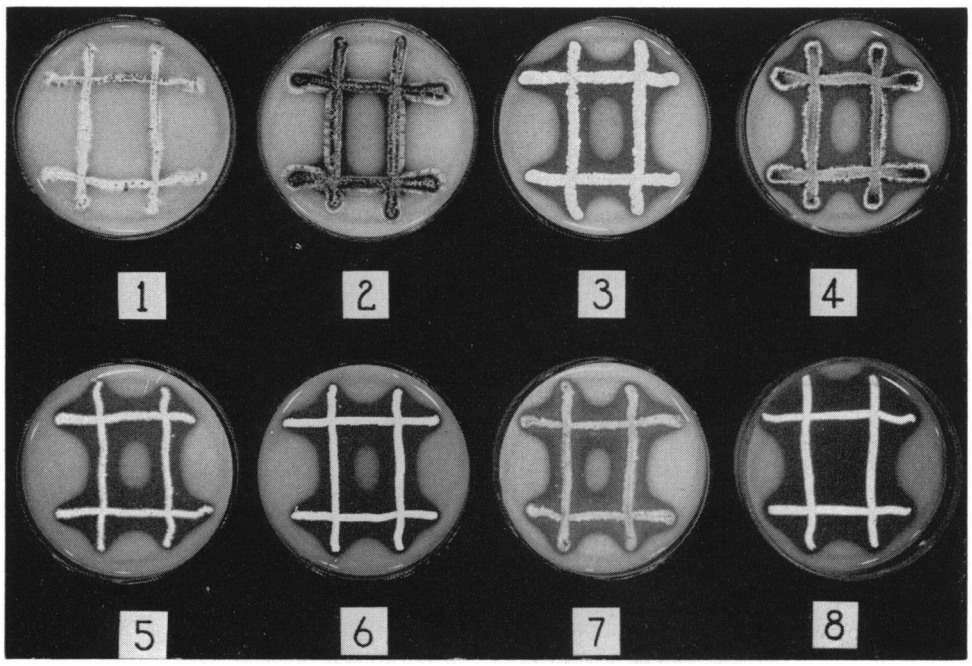

FIG. 4. Cultural and physiologic reactions of six type cultures plus two soil isolates on calcium malate agar. (1) *Streptomyces hygroscopicus* NRRL B-1346. (2) *S. hygroscopicus* NRRL B-1503. (3) *S. hygroscopicus* soil isolate AB-623. (4) *S. hygroscopicus* Baarn strain. (5) *S. endus* NRRL B-2339. (6) *S. platensis* NRRL B-2364. (7) *S. hygroscopicus* ATCC #10976. (8) *S. hygroscopicus* soil isolate AB-965.

of *S. hygroscopicus*, sporulation is very light although aerial mycelium is abundant. Sporulation increases in *S. platensis*, *S. endus*, and the soil isolates, and becomes very abundant in the Baarn strain of *S. hygroscopicus*. The hygroscopic character was poorly represented on Sabouraud's agar and not apparent to any degree except with the Baarn strain and soil isolate AB-965.

On calcium malate agar, the *S. hygroscopicus* group showed considerable variation in the clearing of the

malate as is evidenced in figure 4, ranging from almost none with the NRRL B-1346 strain of *S. hygroscopicus* to the large zone shown with the soil isolate AB-965. Various strains also differed quite remarkably on this medium in both growth habit and in sporulation. Most were restricted and with very sparse sporulation; however, the NRRL B-1503 and the Baarn strains of *S. hygroscopicus* were somewhat spreading and sporulated abundantly. The B-1503 strain was one of the few which developed the hygroscopic feature to any degree on this medium.

Variability in the *S. hygroscopicus* complex is exemplified still further through a consideration of the carbon source utilization patterns exhibited by the group. In table 4, glycerol, mannose, D (+) trehalose, mannitol, and D (+) levulose are shown to be utilized readily by almost all strains; D (+) xylose was utilized by all, but poorly by over half the strains; raffinose by all except the NRRL B-1503 strain of *S. hygroscopicus*; sodium citrate by all except the NRRL B-1503 and the ATCC strains of *S. hygroscopicus*; and lactose by all except one soil isolate (Strain AB-623). Utilization of sucrose, sorbitol, D (−) arabinose, L-rhamnose, and erythritol was variable among the different strains, that is some strains utilized these sources well, while others only poorly or not at all. Inulin and sorbose were generally poor carbon sources, inulin being utilized by only *S. endus* and two soil isolates (AA-398 and A-9822), while sorbose was not utilized by any of the strains studied.

A similar picture of strain variation is obtained from an examination of the nitrogen source utilization patterns in table 5. Alanine, aspartic acid, and histidine were utilized well by most strains; glutamine, glycine, arginine, and proline likewise were utilized well by most strains, but not at all by a few; valine, leucine, and phenyl-alanine were utilized well by many strains, but only poorly or not at all by others; urea, methionine, glutamic acid, and $NaNO_3$ offered even more variable utilization patterns, in that poor or non-utilization was the rule; the ultimate in this direction is shown with $(NH_4)_2PO_4$ and $NaNO_2$ in which the former was utilized by only four soil isolates and the latter by none of the strains.

Discussion

We have attempted to point out the comparative degree of constancy with which the 18 cultures used in this study were observed to display the following three characteristics which we regard as fundamentally those of *S. hygroscopicus*: (1) Sporiferous hyphae terminating in tight spirals of a few to many turns, plus a clustering of such sporiferous structures along subtending hyphae; (2) the brownish-gray spore color (*Mouse Gray* to *Benzo Brown* of Ridgway, 1912) displayed *en masse* by all strains on media which promote abundant sporulation; and (3) the distinctive hygroscopic character which all strains were observed to develop on some agar media. Other characteristics, such as spore size and shape, nature and color of substrate growth on agar media, presence or absence of soluble pigments, presence or absence of exudates, behavior on potato or carrot plugs, cellulose decomposition and so forth, were found to be so variable as to offer considerable difficulty in their use as species criteria. The two type cultures of *S. endus* and *S. platensis* were observed to fit the three characteristics set forth above, while showing no greater variability in other characteristics than did the four *S. hygroscopicus* types and the group of soil isolates studied. For this reason we again point out that these two described "species" are not sufficiently distinct from the *S. hygroscopicus* complex to merit separate species status.

Acknowledgment

The authors wish to thank Marie Hauck for her technical assistance during this study.

Summary

A study was made of the range of variation in certain cultural and physiologic characteristics as displayed by *Streptomyces hygroscopicus*. The characteristics which were most constant in all strains studied were the brownish-gray (*Mouse Gray* to *Benzo Brown* of Ridgway, 1912) color of the spores *en masse*, the tightly wound coils of the spore-bearing hyphae, and the characteristic black hygroscopic areas which all strains developed to some degree on certain agar media.

More variable were such features as color of substrate mycelium and colony reverse, presence or absence of diffusible pigments, ability to clear calcium malate agar, behavior on litmus milk, gelatin liquefaction, carbon and nitrogen source utilization and so forth.

Studies made simultaneously with type cultures of *Streptomyces endus* and *Streptomyces platensis* revealed remarkable similarity to the general *S. hygroscopicus* group. This parallelism suggests that these two organisms might more appropriately be regarded as variants of the *S. hygroscopicus* "complex" than as separate species.

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A Source of Coliforms in Frozen Concentrated Orange Juice

Fruit Surface Contamination

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Several times during past years the attention of freezers of orange juice, both single-strength and concentrate, has been called forcibly to the presence of coliform bacteria in their products. Consequently, workers in industry, university, and government laboratories have undertaken investigations on incidence, sources, and significance of the bacteria in frozen citrus products.

Wolford and Berry (1948a) demonstrated that damaged or "soft rot" oranges may be a source of coliform bacteria when they found that juices produced from unsound fruit contained many times as many microorganisms, including coliforms, as did juices produced from apparently sound processing-grade fruit. These same investigators (1948b) found that coliform bacteria could be recovered from slime which accumulated on the surfaces of fruit-handling equipment, especially equipment which remained moist for extended periods. In more recent years improved sanitation of fruit-handling equipment in citrus plants has gone a long way toward eliminating slime deposits as potential sources of coliforms.

Wolford (1950) demonstrated that *Aerobacter*, intermediates, and *Escherichia coli* could survive as long as 43 weeks in frozen single-strength orange juice stored at −10 F. Patrick (1953) found *E. coli* among a collection of over 100 cultures isolated from damaged oranges and from frozen concentrated orange juice. Patrick (1951) also investigated sources of coliforms in orange juice and found no coliforms on surfaces of, or in juice from fruit sampled in groves; but he found these bacteria on scale insects, fruit flies, and damaged fruit. G. L. Hays, American Can Company, Maywood, Illinois (*personal communication*), however, found that *E. coli* could be isolated from the surfaces of oranges when groves from which the fruit was sampled were watered by overhead irrigation with raw lake water.

Investigations of sources of coliforms in frozen concentrated orange juice produced in California have been made. The present paper covers one phase of these studies, that is, the incidence of coliform bacteria on the surfaces of oranges sampled at various points in groves, packing houses, and processing plants.

MATERIALS AND METHODS

Collection of fruit. In this study, fruit was sampled from trees in the following manner: A sterilized paper bag was held under the orange and the fruit was clipped from the tree with sterilized clippers and allowed to fall into the bag. Fruit was sampled from three locations on the trees. These included inside the tree skirt within 3 feet above ground, outside the tree skirt within 3 feet above ground, and 6 feet or more above ground.

At other sampling points, sterilized tongs were used to transfer fruit to sterilized paper bags. These points included field boxes in groves, unwashed fruit, washed fruit, and processing-grade fruit from packing houses. Samples at the products plants were taken

[1] The author is now stationed in another laboratory of the same Branch in Puyallup, Washington.