THE UTILIZATION OF CARBON COMPOUNDS BY SOME ACTINOMYCETALES AS AN AID FOR SPECIES DETERMINATION

T. G. PRIDHAM AND DAVID GOTTLIEB

University of Illinois, Urbana, Illinois

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Attention has recently been focused on the identification of the actinomycetes because of the ability of some members of this group to produce antibiotics. The determinative procedures now in use are based primarily on morphology, pigmentation, and growth characteristics on media composed of complex natural substrates. With such media one can often observe a variety of pigments and colony types produced by the same organism. In inexperienced hands, this leads to difficulties in interpretation of results, and one is often forced to designate a particular isolate as one of several species rather than one distinct species. To aid in the identification of a group of antibiotic-producing actinomycetes under study, an intensive investigation of their utilization of carbon compounds in chemically defined media was undertaken.

Prior to the current investigations a number of workers had studied the utilization of carbon compounds by some species in this group. Beijerinck (1900), Sames (1900), Caminiti (1913), Fousek (cited by Waksman, 1919), Krainsky (1914), and Gray and Thornton (1928) each tested a few materials and found that various members of this group could grow on media containing different carbon sources. More extensive tests on carbon compounds were made by Salzmann (1907), Munter (1913), and Waksman (1919). Recently Taylor and Decker (1947) showed that various plant pathogenic actinomycetes could be separated from nonpathogenic isolates on the basis of their reactions on carbon compounds, as well as other criteria. The studies of Cochrane (1947) also indicated that different species in the genus *Actinomyces* might be separated on the basis of acid production.

METHODS

Routine identifications. Gram reactions were determined, condidial and hyphal dimensions were measured, and the growth characteristics and responses of the various isolates were observed on the following media; nitrate broth, tryptone broth, starch agar, Kligler's double sugar medium, gelatin, litmus milk, tyrosine broth, nutrient broth, D-glucose agar, Czapek's sucrose agar, calcium *n* butyrate agar, Dorset's egg medium, Loeffler's serum medium, potato slants, and carrot slants. Insofar as possible, all media prepared and procedures followed were those recommended in *A Manual of Methods for Pure Culture Study of Bacteria* (1946), or cited in *Bergey's Manual of Determinative Bacteriology* (1939, 1946). Some of the results thus obtained were used in species identification of each of the

isolates, following the key in *Bergey's Manual* (1946).[1] The identities of the different acquisitions are recorded in table 1 and some of the more important characteristics in table 2.

Reaction on chemically defined media. Stock cultures of the isolates were maintained on a synthetic medium containing either D-glucose or starch as a carbon source, or in nutrient broth (tryptone 0.5 per cent, yeast extract 0.3 per cent). The utilization of carbon compounds was tested on the following basal medium:

$(NH_4)_2SO_4$	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4 \cdot 7H_2O$	1.00 g
$CuSO_4 \cdot 5H_2O$	0.0064 g
$FeSO_4 \cdot 7H_2O$	0.0011 g
$MnCl_2 \cdot 4H_2O$	0.0079 g
$ZnSO_4 \cdot 7H_2O$	0.0015 g
Difco agar	15.00 g
Distilled water	1,000 ml

This medium was adjusted to pH 6.8 to 7.0, tubed in 9½-ml amounts, and autoclaved. After cooling to about 45 C, sterile aqueous solutions of the carbon compounds were added to give the proper concentration. The carbohydrates, polyhydric alcohols, DL-inositol, and salicin were added such that that the final concentration was 1 per cent, the phenols 0.1 per cent, and the sodium salts of the organic acids 0.15 per cent. Those materials sufficiently soluble in water were sterilized by filtration through Seitz EK filter pads. Some compounds (dextrin, starch, dulcitol, DL-inositol, and salicin) that were relatively insoluble or did not filter well were added directly to the basal medium in the proper concentration prior to tubing and sterilization. After addition of the carbon sources, the tubes were slanted, allowed to solidify, and incubated to determine sterility.

Inocula were prepared by growing the isolates in nutrient broth (Difco tryptone 0.5 per cent, yeast extract 0.3 per cent) at room temperature for 2 weeks. The liquid was decanted, and the remaining mycelium was washed with sterile distilled water, transferred to 100 ml of sterile distilled water, and thoroughly shaken. One-half ml of this suspension was allowed to flow over the surface of the test medium. Controls consisting of tubes of basal medium alone were always inoculated with each run. Observations for the presence or absence of growth were made after 10 days' incubation at 26 C. The ability of the different compounds to support growth was tested in 2 to 14 replicate tubes for each material and the experiments were usually repeated from 2 to 9 times during an interval of 18 months.

RESULTS

Identification of isolates. The characteristics of those cultures that were received under specific names agreed with our determinations except for minor

[1] The authors are greatly indebted to Dr. R. S. Breed, New York Agricultural Experiment Station, and to Dr. S. A. Waksman, New Jersey Agricultural Experiment Station, for kindly furnishing this information.

differences (tables 1 and 2). The unknown isolates, however, were harder to identify because of difficulties in the evaluation of pigment production and colony types. Some of the characteristics so determined could not be appropriately

TABLE 1

Source and identity of cultures

ISOLATE	SOURCE	IDENTITY
Streptomyces griseus A2	S. A. Waksman, Rutgers Univ.	As received
" " B	G. H. Savage, The Upjohn Co.	" "
" " 72	H. W. Anderson, Univ. of Ill.	" "
" " 434	A. J. Whiffen, The Upjohn Co.	" "
S. lavendulae 3440-14	S. A. Waksman, Rutgers Univ.	" "
A. "	ATCC	" "
A-56, probable strain of *S. lavendulae*	A. J. Whiffen, The Upjohn Co.	*S. lavendulae*
A-151 probable strain of *S. lavendulae*	" " " " " "	" "
A-10 variant of *S. lavendulae*	H. W. Morton, Univ. of Penn.	" "
S. antibioticus	S. A. Waksman, Rutgers Univ.	*S. antibioticus*
A. scabies	W. D. Thomas, Col. State Coll.	As received
A. scabies 3352	ATCC	" "
A. albus 618	ATCC	" "
A-105, *A. erythreus, fradiae, albosporeus*, or *californicus*	H. E. Morton, Univ. of Penn.	*S. fradiae*, or *albosporeus*
83D, unidentified	H. W. Anderson, Univ. of Ill.	*S. flavovirens*
211, unidentified	C. H. Meredith, Iowa State Coll.	*S. erythrochromogenes*
8-48, unidentified	D. Gottlieb, Univ. of Ill.	*S. griseolus, rutgernensis*, or *halstedii*
8-12, unidentified	" " " " "	*S. fradiae* or *albosporeus*
8-80, unidentified	" " " " "	*S. fradiae, albo-flavus*, or *californicus*
8-96, unidentified	" " " " "	*S. fradiae, albo-flavus*, or *californicus*
8-44, unidentified	" " " " "	*S. lavendulae*, or *reticuli*
S. violaceous-ruber 3030	S. A. Waksman, Rutgers Univ.	As received
Nocardia gardneri 3449	" " " " "	" "
N. asteroides 3308	" " " " "	" "
N. polychromogenes 3409	" " " " "	" "
Micromonospora sp. 3450	" " " " "	*M. fusca*
" " 3451	" " " " "	" "

fitted into the key. For this reason some of the isolates were given more than one possible species designation (table 1).

Growth responses to carbon sources in chemically defined media. Generally, the

TABLE 2

Cultural characteristics of various actinomycetes, †*

ISOLATE	H_2S	GELATIN LIQ.	TYROSINASE	CZAPEK'S MEDIUM	DORSET'S EGG MEDIUM	LOEFFLER'S SERUM	STARCH MEDIUM	POTATO
S. griseus (all strains)	−	+	−	P, A, wh to c	G, fl, gr-	G, fl, gr-wh to brn	G, A, gr-wh	G, A, wh
S. lavendulae (all strains) *S. antibioticus*	+	+	+	F, A, lav to wh	F, S, dk brn	F, Sm, dk brn	G, A, lav-wh G, Sm, brn	G, A, lav
A. scabies	−	+	−	G, A, wh	G, A, wh	G, A, wh	G, A, wh	G, A, wh
A. scabies 3352	+		+	P, A, c	G, Sm, gr- wh	G, S, gr-wh	G, A, Sm, gr	G, A, wh
A. albus 618	−		−		G, Sm, wh	G, Sm, wh	G, A, Sm, wh	
S. flavovirens 83D9	+	+	+	F, A, wh-gr	G, Sm, yel	G, Sm, yel-gre	G, A, gr-wh	G, A, gr-gre
S. erythrochromogenes 211	+	+	+	F, A, wh	G, Sm, rd-lav	G, Sm, gr-wh	G, A, Sm, lav-wh	G, A, wh-rd
A-105	−			F, A, Sm, lav-wh rd rev	G, A, Sm, rd-wh	G, A, Sm, wh-rd	G, A, lav-wh	G, A, Sm, wh-rd
S. violaceus-ruber 3030	−		+	G, A, Sm, wh-bl	G, Sm, wh	G, Sm, wh	G, A, Sm, wh-rd	G, A, wh
8-12	−	+	−	G, A, wh- lav or rev	G, A, wh	G, A, wh	G, A, Sm, wh-lav	G, A, wh rev rd
8-80 and 8-96	+	+	−	G, A, lav- wh to or- yel red rev	G, A, wh-yel	G, A, wh to yel	G, A, lav-wh to or-yel rev rd	G, A, wh-gr-rd rev or-rd
8-48	−	+	+	G, A, gr to wh	G, A, gr to wh	G, A, wh to gr	G, A, wh to gr	G, A, gr to wh
8-44	+	+	+	G, A, Sm, lav	G, A, wh to gr	G, Sm, dk brn	G, A, lav-wh	G, A, gr
N. asteroides 3308	−	−	−		G, Sm, yel or, rd	G, Sm, yel, rd, or	G, Sm, p, or	F, Sm, c or
N. polychromogenes 3409	−	−	−		G, Sm, p, yel, or rd	G, Sm, or yel, rd	F, Sm, p, or, rd, wh	F, Sm, p, wh-rd
N. gardneri 3449	−	+	−	F, Sm, wh	G, Sm, gr-gre	G, Sm, gre-gr	G, Sm, wh	F, Sm, wh
M. fusca 3450 and 3451	−	+	−	F, Sm, p, wh	G, Sm, p, or to blk	G, Sm, p, or to blk	F, Sm, p, or to blk	F, Sm, yel-brn

* All cultures reduced nitrates; none produced indole, and only one culture, *S. flavovirens*, liquefied Dorset's medium.

† Growth: G—good, F—fair, P—poor, A—aerial hyphae, Sm—smooth, p—punctiform, fl—flat.

Pigment: wh—white, gr—gray, lav—lavender, brn—brown, or—orange, blk—black, bl—blue, yel—yellow, rd—red, gre—green, c—colorless, rev—reverse of colony.

growth responses on the different media were well defined and were similar in the various experiments performed over a period of 18 months. The ability of the different isolates to grow on the test media varied considerably in different species (table 3). In every case, the control tubes showed very little or no growth, and those tubes in which the isolates could effectively utilize the particular carbon source had very profuse growth. Occasionally, only very slight growth was observed with some materials, indicating that the particular compound was not an adequate source of carbon in that concentration, or that the materials used contained traces of other compounds. It is significant that all the species tested could be differentiated from one another on the basis of their growth on the various carbon sources alone (table 3) and these differences could be substantiated by other physiological characteristics (table 2).

The four strains of *S. griseus*, although obtained from different sources, gave identical reactions, thus indicating a marked uniformity in the different isolates of this species. Similarly the two known strains of *S. lavendulae* (*Actinomyces lavendulae* and 3440-14) and isolate A-151, identified as *S. lavendulae*, likewise gave identical reactions with all but one of the compounds, sodium acetate. Of the remaining isolates tentatively identified as *S. lavendulae*, A-10 and A-56 differed from the accepted strains of *S. lavendulae* in the utilization of only two of the compounds.

Particular interest was placed on the results obtained with isolates 83D and 8-44. Isolate 83D produces actinomycin,[2] an antibiotic elaborated by *S. antibioticus* and other *Streptomyces* sp. (Waksman and Woodruff, 1940; Waksman, Geiger, and Reynolds, 1946). This isolate, which was identified as *S. flavovirens*, differs from *S. antibioticus* on six of the carbon sources tested as well as on the routine media; thus two different known species produce the same antibiotic. Isolate 8-44 resembles *S. lavendulae* or *S. reticuli* when determined by the usual identification procedures, but appears quite different from *S. lavendulae* in its ability to utilize some pentoses and sodium acetate. In addition, 8-44 produces only the antibiotic, chloromycetin, whereas *S. lavendulae* produces streptothricin.

Isolate A-105 resembles to a large degree isolates 8-12, 8-80, and 8-96, by the usual keys, but differs from them in the utilization of four of the compounds tested. Both isolates A-105 and the 8-80 group[3] produce antibiotics with some similar properties, but these antibiotics have not been completely characterized.

The two strains of *S. scabies*, which appeared different when the routine procedures for identification were used, also exhibited a number of differences in ability to utilize the various carbon sources tested. Twelve differences on the test media combined with the differences observed during preliminary identification might indicate that one of these isolates is not a true *S. scabies*, or that strain differences in this species are very marked.

With the exception of *N. gardneri*, the species of *Nocardia* and *Micromonospora*

[2] Unpublished results of H. W. Anderson and H. E. Carter, University of Illinois.

[3] Unpublished results of David Gottlieb, P. K. Bhattacharyya, and H. E. Carter, University of Illinois.

TABLE 3

Growth of test organisms on synthetic medium plus various carbon sources,†*

COMPOUND	*S. griseus*				*S. lavendulae*					ISOLATE					*S. antibioticus*	*S. flavovirens* 83D	ISOLATE		*Nocardia gardneri* 3449	*S. scabies*		*S. albus* 618	*S. violaceus-ruber* 3030
	72	A2	B	434	A. lav.	3440-14	A-151	A-56	A-10	8-44	8-80	8-96	8-12	A-105			8-48	211		*A. scabies*	3352		
L-Xylose	+	+	+	+	—	?	(—)	—	—	+	+	+	+	—	+	+	+	+	+	+	+	+	+
L-Arabinose	(—)	—	(—)	(—)	—	?	(—)	—	—	+	+	+	+	—	+	+	+	+	+	+	(—)	+	+
Rhamnose	—	—	—	—	—	—	(—)	—	—	+	+	+	+	+	+	+	+	+	+	+	+	—	+
D-Fructose	+	+	+	+	?	?	+	+	(—)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	(—)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	?	?	?	?	—	—	—	—	—	—	+	+	+	+	(—)	+	?	+	+	+	—	?	?
Maltose	+	+	+	+	+	+	+	—	+	?	+	+	+	+	(—)	+	(—)	+	+	+	+	+	+
Lactose	+	+	+	+	—	—	(—)	—	—	(—)	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	+	(—)	+	+	+	—	—	+
Inulin	(—)	(—)	(—)	(—)	—	—	(—)	(—)	(—)	(—)	?	+	+	+	+	+	—	+	+	+	—	—	—
D-Mannitol	+	+	+	+	(—)	(—)	(—)	(—)	—	(—)	+	+	+	+	+	+	+	+	(—)	+	+	+	+
D-Sorbitol	(—)	(—)	(—)	(—)	(—)	—	(—)	(—)	—	(—)	+	+	+	+	(—)	+	+	(—)	—	+	—	+	+
Dulcitol	—	—	—	—	—	—	(—)	—	—	—	(—)	(—)	+	+			(—)	(—)	—	+	—	+	+
DL-Inositol	(—)	(—)	—	—	(—)	(—)	(—)	(—)	(—)	(—)	+	+	+	+	+	+	+	+	—	—	—	(—)	+
Salicin	+	+	+	+	+	+	+	(—)	+	+	+	+	+	+	(—)	+	+	+	+	+	(—)	(—)	+
Na-acetate	+	+	+	+	(—)	(—)	+	+	+	+	+	+	+	+	+	(—)	+	+	+	+	(—)	+	+
Na-citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	?	(—)	+	?
Na-succinate	+	+	+	+	+	+	+	+	(—)	+	(—)	+	(—)	+		+	(—)	+	+	(—)	—	+	(—)

* All isolates utilized D-glucose, D-mannose, cellobiose, starch, dextrin, and glycerol; none of the isolates utilized phenol, *o*-cresol, *m*-cresol, sodium formate, oxalate, tartrate, or salicylate.

† Key: + = growth and positive utilization; — = no growth, no utilization; (—) = faint growth, probably no utilization; ? = variable reaction, growth positive at times, negative at others.

gave inconclusive results, since these microorganisms did not grow well on the test media.

Using the information provided in table 3, a flow sheet (figure 1) was prepared on which all the antibiotic-producing isolates could be separated on the basis of their growth responses. Other simple tests could be readily fitted into this

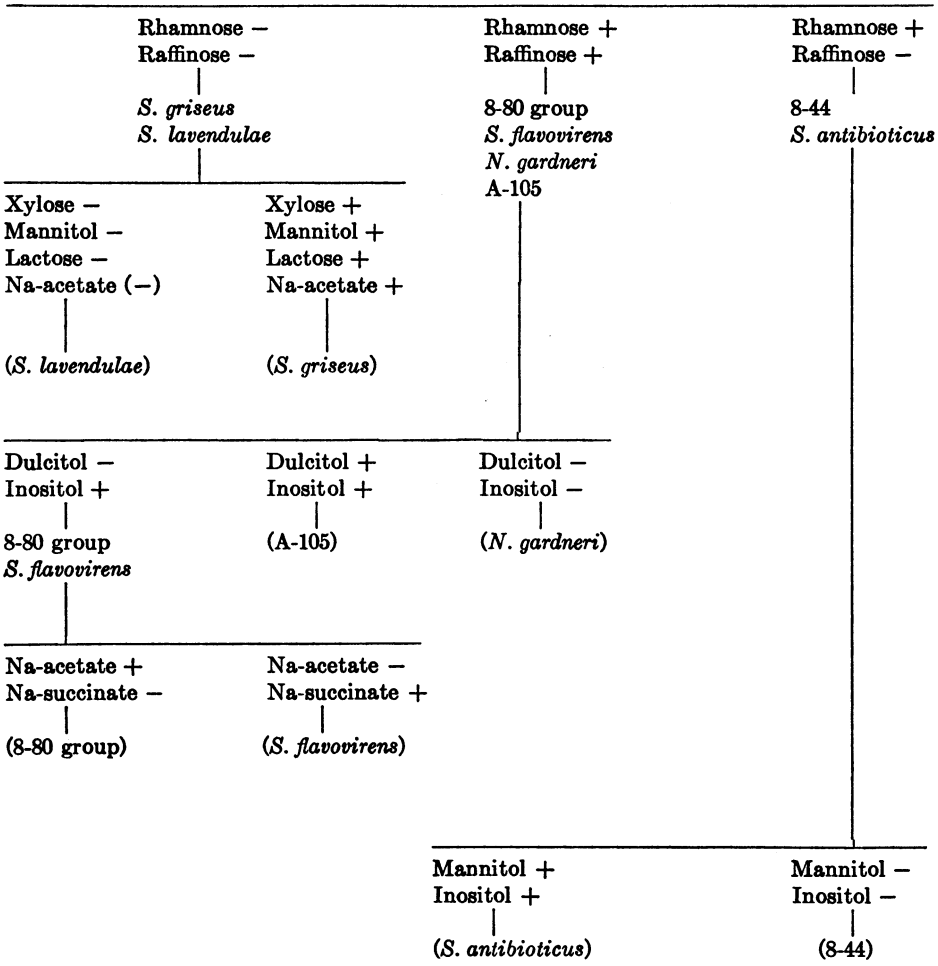

Figure 1. Flow sheet for the separation of antibiotic-producing isolates on the basis of utilization of carbon compounds in a chemically defined medium.

scheme (table 2). Further to substantiate the differences between any two species on the flow sheet other responses from among those listed in table 3 could be chosen. A knowledge of the utilization of carbon compounds in a chemically defined media should be a valuable addition to determinative keys and aid in the identification of members of the *Actinomycetales*, especially the genus *Streptomyces*. It is of special interest when a small group of organisms are concerned

such as the antibiotic-producing actinomycetes; among these most of the species can be separated by such reactions alone.

SUMMARY

Twenty-seven isolates of organisms belonging to the genera *Streptomyces*, *Nocardia*, and *Micromonospora* have been tested for their ability to utilize 33 different carbon compounds as a source of carbon in a chemically defined medium. The results obtained with the antibiotic-producing streptomycetes and others indicate that such reactions can aid species identification. All of the streptomycetes studied were found to utilize D-glucose, D-mannose, starch, dextrin, and glycerol, but not erythritol, phenol, *o*-cresol, *m*-cresol, *p*-cresol, Na-formate, Na-oxalate, and Na-tartrate. Reactions on the other carbon compounds varied with the particular species.

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